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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61R 48/00 | A2

(11) International Publication Number: WO 00/54839

(43) International Publication Date: 21 September 2000 (21.09.00)

(21) International Application Number: PCT/US00/07055

(22) International Filing Date: 15 March 2000 (15.03.00)

(30) Priority Data:
60/124,482 15 March 1999 (15.03.99) US
60/124,388 15 March 1999 (15.03.99) US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: DENDRITIC CELLS TRANSDUCED WITH A WILD-TYPE SELF GENE ELICIT POTENT ANTITUMOR IMMUNE RESPONSES

(57) Abstract

The present invention relates to immunotherapy methods for treating hyperproliferative disease or pathogen-induced diseases in humans. More specifically, the invention is directed, in one embodiment, to methods for treating a subject with a hyperproliferative disease in which the expression of a self gene is upregulated in hyperproliferative cells. In another embodiment, an adenoviral expression construct comprising a self gene under the control of a promoter operable in eukaryotic cells is intradermally administered to said hyperproliferative cells. In another embodiment of the present invention, a pathogen-induced disease in which the pathogen gene expression is increased or altered, is treated by intradermally administered a pathogen gene under the control of a promoter operable in eukaryotic cells. The present invention thus provides immunotherapies for treating hyperproliferative and pathogen diseases by attenuating the natural immune systems CTL response against hyperproliferative cells or overexpressing mutant p53 antigens.

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DESCRIPTION

DENDRITIC CELLS TRANSDUCED WITH A WILD-TYPE SELF GENE ELICIT POTENT ANTITUMOR IMMUNE RESPONSES

BACKGROUND OF THE INVENTION

The present application claims the benefit of U.S. Provisional Application Serial Number 60/124,482 and U.S. Provisional Application Serial Number 60/124,388, both of which were filed on March 15, 1999. The government owns rights in the present invention pursuant to grant number CA61242 from the National Cancer Institute.

A. FIELD OF THE INVENTION

The present invention relates generally to the fields of immunology and cancer therapy. More particularly, it concerns a method of eliciting a cytotoxic T lymphocyte response directed against self gene antigens presented by hyperproliferative cells.

B. DESCRIPTION OF RELATED ART

Normal tissue homeostasis is a highly regulated process of cell proliferation and cell death. An imbalance of either cell proliferation or cell death can develop into a cancerous state (Solyanik *et al.*, 1995; Stokke *et al.*, 1997; Mumby and Walter, 1991; Natoli *et al.*, 1998; Magi-Galluzzi *et al.*, 1998). For example, cervical, kidney, lung, pancreatic, colorectal and brain cancer are just a few examples of the many cancers that can result (Erlandsson, 1998; Kolmel, 1998; Mangray and King, 1998; Gertig and Hunter, 1997; Mougin *et al.*, 1998). In fact, the occurrence of cancer is so high, that over 500,000 deaths per year are attributed to cancer in the United States alone.

The maintenance of cell proliferation and cell death is at least partially regulated by proto-oncogenes. A proto-oncogene can encode proteins that induce cellular proliferation (*e.g., sis, erbB, src, ras* and *myc*), proteins that inhibit cellular

proliferation (*e.g., Rb, p53, NF1* and *WT1*) or proteins that regulate programmed cell death *(e.g., bcl-2)* (Ochi *et al.*, 1998; Johnson and Hamdy, 1998; Liebermann *et al.*, 1998). However, genetic rearrangements or mutations to these proto-oncogenes, results in the conversion of a proto-oncogene into a potent cancer causing oncogene. Often, a single point mutation is enough to transform a proto-oncogene into an oncogene. For example, a point mutation in the p53 tumor suppressor protein results in the complete loss of wild-type p53 function (Vogelstein and Kinzler, 1992; Fulchi *et al.*, 1998) and acquisition of "dominant" tumor promoting function.

Currently, there are few effective options for the treatment of many common cancer types. The course of treatment for a given individual depends on the diagnosis, the stage to which the disease has developed and factors such as age, sex and the general health of the patient. The most conventional options of cancer treatment are surgery, radiation therapy and chemotherapy. Surgery plays a central role in the diagnosis and treatment of cancer. Typically, a surgical approach is required for biopsy and to remove cancerous growth. However, if the cancer has metastasized and is widespread, surgery is unlikely to result in a cure and an alternate approach must be taken. Radiation therapy, chemotherapy and immunotherapy are alternatives to surgical treatment of cancer (Mayer, 1998; Ohara, 1998; Ho *et al.*, 1998). Radiation therapy involves a precise aiming of high energy radiation to destroy cancer cells and much like surgery, is mainly effective in the treatment of non-metastasized, localized cancer cells. Side effects of radiation therapy include skin irritation, difficulty swallowing, dry mouth, nausea, diarrhea, hair loss and loss of energy (Curran, 1998; Brizel, 1998).

Chemotherapy, the treatment of cancer with anti-cancer drugs, is another mode of cancer therapy. The effectiveness of a given anti-cancer drug therapy is often limited by the difficulty of achieving drug delivery throughout solid tumors (el-Kareh and Secomb, 1997). Chemotherapeutic strategies are based on tumor tissue growth, wherein the anti-cancer drug is targeted to the rapidly dividing cancer cells. Most chemotherapy approaches include the combination of more than one anti-cancer drug,

which has proven to increase the response rate of a wide variety of cancers (U.S. Patent 5,824,348; U.S. Patent 5,633,016 and U.S. Patent 5,798,339). A major side effect of chemotherapy drugs is that they also affect normal tissue cells, with the cells most likely to be affected being those that divide rapidly (*e.g.*, bone marrow, gastrointestinal tract, reproductive system and hair follicles). Other toxic side effects of chemotherapy drugs are sores in the mouth, difficulty swallowing, dry mouth, nausea, diarrhea, vomiting, fatigue, bleeding, hair loss and infection.

Immunotherapy, a rapidly evolving area in cancer research, is yet another option for the treatment of certain types of cancers. For example, the immune system identifies tumor cells as being foreign and thus are targeted for destruction by the immune system. Unfortunately, the response typically is not sufficient to prevent most tumor growths. However, recently there has been a focus in the area of immunotherapy to develop methods that augment or supplement the natural defense mechanism of the immune system. Examples of immunotherapies currently under investigation or in use are immune adjuvants (*e.g., Mycobacterium bovis, Plasmodium falciparum*, dinitrochlorobenzene and aromatic compounds) (U.S. Patent 5,801,005; U.S. Patent 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998), cytokine therapy (*e.g.*, interferons α, β and γ; IL-1, GM-CSF and TNF) (Bukowski *et al.*, 1998; Davidson *et al.*, 1998; Hellstrand *et al.*, 1998) gene therapy (*e.g.*, TNF, IL-1, IL-2, p53) (Qin *et al.*, 1998; Austin-Edward and Villaseca, 1998; U.S. Patent 5,830,880 and U.S. Patent 5,846,945) and monoclonal antibodies (*e.g.*, anti-ganglioside GM2, anti-HER-2, anti-p185) (Pietras *et al.*, 1998; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311).

As mentioned above, proto-oncogenes play an important role in cancer biology. For example, Rb, p53, NF1 and WT1 tumor suppressors, are essential for the maintenance of the non-tumorogenic phenotype of cells (reviewed by Soddu and Sacchi, 1998). Approximately 50% of all cancers have been found to be associated with mutations of the p53 gene, which result in the loss of p53 tumor suppressor properties (Levine *et al.*, 1991; Vogelstein and Kinzler, 1992; Hartmann *et al.*, 1996a;

Hartmann *et al.*, 1996b). Mutations in the p53 gene also result in the prolongation of the p53 half-life in cells and the overexpression of p53 protein. In normal cells, p53 is undetectable due to its high turnover rate. Thus, p53 overexpression in cancerous cells results in multiple immunogenic p53 epitopes which can be used in immunotherapy. The high incidence of cancer related to mutations of the p53 gene has prompted many research groups to investigate p53 as a route of cancer treatment via gene replacement. The proto-oncogenes sis, erbB, src, ras and myc, encoding proteins that induce cellular proliferation, and the proto-oncogenes of the Bcl-2 family that regulate programmed cell death also play important roles in the non-tumorogenic phenotype of cells.

A few also have explored the use of p53 in immunotherapy. For example, in an *in vitro* assay, p53 mutant peptides capable of binding to HLA-A2.1 and inducing primary cytotoxic T lymphocyte (CTL) responses were identified (Houbiers *et al.*, 1993). In a study in which synthetic p53 mutant and wild-type peptides were screened for immunogenicity in mice, it was observed that only mutant p53 epitopes were capable of eliciting a CTL response (Bertholet *et al.*, 1997). In contrast, the immunization of BALB/c mice with bone marrow-derived dendritic cells (DC) in the presence of GM-CSF/IL-4 and prepulsed with the H-2Kd binding wild-type p53 peptide (232-240) was observed to induce p53 anti-peptide CTL response (Ciernik *et al.*, 1996; Gabrilovich *et al.*, 1996; Yanuck *et al*, 1993; DeLeo, 1998; Mayordomo *et al.*, 1996). Further, the intradermal and intramuscular injection of naked plasmid DNA encoding human wild-type p53 and the intravenous injection of human wild-type p53 presented by a recombinant canarypox vector have been successful in the destruction of tumors (Hurpin *et al.*, 1998).

Despite the foregoing, there currently exist no methods of self gene-based immunotherapy capable of utilizing wild-type self genes to generate an antitumor immune response specific for a variety of cells overexpressing different mutant self proteins. This would permit the treatment of any cancerous or pre-cancerous cell associated with increased or altered expression of the self gene. Further, it would

eliminate the need to identify the site of self gene mutation in each patient and generate customized self gene mutant peptides for immunotherapy. Thus, the need exists for an immunotherapy that is capable of attenuating or enhancing the natural immune systems CTL response against hyperproliferative cells with increased or altered expression of mutant self gene antigens.

SUMMARY OF THE INVENTION

Therefore, there exists a need for an immunotherapy that is capable of augmenting the natural immune systems CTL response against hyperproliferative cells or pathogen infected cells expressing an altered self gene antigen or pathogenic antigen, respectively. The present invention also provides a method of eliciting a cytotoxic T lymphocyte response directed against p53 antigens presented by hyperproliferative cells. In one embodiment of the invention, there is provided a method for treating a subject with a hyperproliferative disease.

The treatment of a hyperproliferative disease in the present invention comprises the steps of identifying a subject with a hyperproliferative disease, characterized by alteration or increased expression of a self gene product in at least some of the hyperproliferative cells in the patient. Following identification of a subject with a hyperproliferative disease, an expression construct comprising a self gene under the control of a promoter operable in eukaryotic dendritic cells is intradermally administered to the subject. The self gene product is expressed by dendritic cells and presented to immune effector cells, thereby stimulating an anti-self gene product response.

In one embodiment, the self-gene product is an oncogene, wherein the oncogene may be selected from the group consisting of tumor suppressors, tumor associated genes, growth factors, growth-factor receptors, signal transducers, hormones, cell cycle regulators, nuclear factors, transcription factors and apoptic factors. In preferred embodiments, the tumor suppressor is selected from the group

consisting of Rb, p53, p16, p19, p21, p73, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC , PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2 and WT-1. In preferred embodiments, the tumor suppressor is p53. In preferred embodiments, the growth-factor receptor is selected from the group consisting of FMS, ERBB/HER, ERBB-2/NEU/HER-2, ERBA, TGF-β receptor, PDGF receptor, MET, KIT and TRK. In preferred embodiments, the signal transducer is selected from the group consisting of SRC, ABl, RAS, AKT/PKB, RSK-1, RSK-2, RSK-3, RSK-B, PRAD, LCK and ATM. In preferred embodiments, the transcription factor or nuclear factor is selected from the group consisting of JUN, FOS, MYC, BRCA1, BRCA2, ERBA, ETS, EVII, MYB, HMGI-C, HMGI/LIM, SKI, VHL, WT1, CEBP-α, NFKB, IKB, GL1 and REL. In preferred embodiments, the growth factor is selected from the group consisting of SIS, HST, INT-1/WT1 and INT-2. In preferred embodiments, the apoptic factor is selected from the group consisting of Bax, Bak, Bim, Bik, Bid, Bad, Bcl-2, Harakiri and ICE proteases. In preferred embodiments, the tumor-associated gene is selected from the group consisting of CEA, mucin, MAGE and GAGE.

The expression construct may be a viral vector, wherein the viral vector is an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector, an alphavirus vector, or a herpesviral vector. In preferred embodiments, the viral vector is an adenoviral vector.

In certain embodiments, the adenoviral vector is replication-defective. In another embodiment, the replication defect is a deletion in the E1 region of the virus. In certain embodiments, the deletion maps to the E1B region of the virus. In other embodiments, the deletion encompasses the entire E1B region of the virus. In another embodiment, the deletion encompasses the entire E1 region of the virus.

In one embodiment of the present invention, the promoter operable in eukaryotic cells may be selected from the group consisting of CMV IE, dectin-1, dectin-2, human CD11c, F4/80 and MHC class II. In preferred embodiments, the

promoter is CMV IE. In another embodiment the expression vector further comprises a polyadenylation signal.

It is contemplated, in one embodiment of the present invention, that the hyperproliferative disease is cancer, wherein the cancer may be selected from the group consisting of lung, head, neck, breast, pancreatic, prostate, renal, bone, testicular, cervical, gastrointestinal, lymphoma, brain, colon, skin and bladder. In other embodiments, the hyperproliferative disease is non-cancerous and may be selected from the group consisting of rheumatoid arthritis (RA), inflammatory bowel disease (IBD), osteoarthritis (OA), pre-neoplastic lesions in the lung and psoriasis.

In other embodiments, the subject treated for a hyperproliferative disease is a human. It is contemplated in certain embodiments administering to the subject at least a first cytokine selected from the group consisting GM-CSF, IL-4, C-KIT, Steel factor, TGF-β, TNF-α and FLT3 ligand. In yet another embodiment, a second cytokine, different from the first cytokine, is administered to the subject. In another embodiment, the cytokine is administered as a gene encoded by the expression construct. In other embodiments, the immune effector cells are CTLs.

Also contemplated in the present invention is intradermal administration of the expression construct by a single injection or multiple injections. In one embodiment, the injections are performed local to a hyperproliferative or tumor site. In another embodiment, the injections are performed regional to a hyperproliferative or tumor site. In still another embodiment, the injections are performed distal to a hyperproliferative or tumor site. It is further contemplated, that the injections are performed at the same time, at different times or via continuous infusion.

The present invention comprises a method for inducing a p53-directed immune response in a subject comprising the steps of obtaining dendritic cells from a subject, infecting the dendritic cells with an adenoviral vector comprising a p53 gene under the control a promoter operable in eukaryotic cells and administering the adenovirus-

infected dendritic cells to the subject, whereby p53 expressed in the dendritic cells is presented to immune effector cells, thereby stimulating an anti-p53 response.

In another aspect of the present invention, there is provided a method for treating a pathogen-induced disease in a subject comprising the steps of identifying a subject with a pathogen-induced disease characterized by alteration or increased expression of a pathogen gene product in at least some of the pathogen-induced cells in the patient and intradermally administering to the subject an expression construct comprising a pathogen gene under the control of a promoter operable in eukaryoticdendritic cells, whereby the pathogen gene product is expressed by dendritic cells and presented to immune effector cells, thereby stimulating an anti-pathogen gene product response. In one embodiment, the dendritic cells are obtained from peripheral blood progenitor cells. In another embodiment, multiple injections of adenovirus-infected dendritic cells is contemplated.

In one embodiment of the present invention, the pathogen may be selected from the group consisting of bacterium, virus, fungus, parasitic worm, amoebae and mycoplasma. In certain embodiments, the bacterium may be selected from the group consisting of richettsia, listeria and histolytica. In other embodiments the virus may be selected from the group consisting of HIV, HBV, HCV, HSV, HPV, EBV and CMV. In yet another embodiment, the fungus may be selected from the group consisting of hitoplasma, coccidis, immitis, aspargillus, actinomyces, blastomyces, candidia and streptomyces.

In certain embodiments for the treatment of a pathogen-induced disease, the expression construct is a viral vector and may be selected from the group consisting of an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector, an alphavirus vector, or a herpesviral vector. In a preferred embodiment, the viral vector is an adenoviralvector, wherein said adenoviral vector is replication-defective. In one embodiment, the replication defect is a deletion in the E1 region of the virus. In other embodiments, the deletion maps to the E1B

region of the virus. In yet other embodiments, the deletion encompasses the entire E1B region of the virus. In still other embodiments, the deletion encompasses the entire E1 region of the virus.

The promoter operable in eukaryotic cells may be selected from the group consisting of CMV IE, dectin-1, dectin-2, human CD11c, F4/80 and MHC class II. In preferred embodiments, the promoter is CMV IE. In certain embodiments, the expression vector further comprises a polyadenylation signal.

It is contemplated in embodiments where the expression construct is delivered intradermally, that administration may be by injection. In other embodiments, intradermal administration comprises multiple injections. It is contemplated in the present invention, that the injections are performed local, regional or distal to the pathogen-induced disease site.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A, FIG. 1B, and FIG. 1C. Expression of p53 protein in DC infected with Ad-p53. DCs generated from bone marrow were infected with 100 MOI Ad-c or Ad-p53 for 48 h, washed, fixed, permeablized and stained with anti-p53 antibody and analyzed. Non-specific staining - Ad-p53 infected DCs stained only with secondary antibody. Ad-c and Ad-p53, DC infected with corresponding virus stained with anti-p53 antibody. Typical results of one of three studies performed are shown.

FIG. 2A, FIG. 2B and FIG. 2C. Ad-p53 transduced DCs induce anti-p53 immune responses. FIG 2A. CTL response. Mice were immunized twice with DC

infected with either Ad-c (Ad-c DC) or with Ad-p53 (Ad-p53 DC) (iv injections). Ten days after the last immunization, T cells from these mice were restimulated with Ad-p53 DC and a CTL assay was performed. P815-Ad and P815-Ad-p53 targets were prepared by overnight incubation of P815 cells with adenovirus at MOI 100 pfu/ml. Mean±SE of cytotoxicity from four studies is shown. FIG. 2B. CTL responses against MethA mouse tumor sarcoma cells (expressing mutant mouse p53). Mice were immunized, T cells were restimulated and CTL assay was performed exactly as described in FIG. 2A. Target MethA sarcoma cells were pre-incubated with 50 U/ml IFNγ for three days prior the assay. Two studies with the same results were performed. FIG. 2C. T cell proliferation. Mice were immunized as described in FIG. 2A. T cells were isolated and cultured in triplicates with either control untreated DC, Ad-c DC or Ad-p53 DC. ^{3}H-thymidine uptake was measured on day 3. Mean ± SE of thymidine incorporation from two studies is shown.

FIG. 3A and FIG. 3B. Immunization with Ad-p53 protects from tumor challenge. Mice were immunized as described in FIG. 2A. Ten days after the second immunization, mice were challenged with 2×10^5 D459 (mouse cell expressing human p53) cells or with 6×10^5 MethA sarcoma cells. In studies with D459 cells, each group included 20 mice, in studies with MethA sarcoma they included 11 mice. Differences between groups were statistically significant ($p<0.05$).

FIG. 4. Treatment with Ad-p53 DC slowed the growth of established tumors. 2×10^5 D459 cells were inoculated sc into the shaved backs of mice. Treatment with 2×10^5 Ad-c or Ad-p53 DC was initiated when tumor became palpable (day 5). DC were injected on day 5, 9 and 13. Mice in the control group were sacrificed on day 31 due to bulky tumors, mice that received treatment with Ad-p53 DC were sacrificed on day 49. Ten mice per group were treated. Mean ± SE is shown.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention contemplates the treatment of hyperproliferative disease by identifying patients with a hyperproliferative disease in which self gene expression is increased or altered in these hyperproliferative cells. The treatment of such a hyperproliferative disease in one embodiment involves the intradermal administration of a p53 expression construct to dendritic cells, which subsequently present the processed p53 wild-type antigens to immune effector cells. The immune effector cells then mount an anti-p53 response, resulting in the destruction or lysis of hyperproliferative cells presenting mutant p53 antigen. In another embodiment, dendritic cells are obtained from a patient in which p53 expression is upregulated in hyperproliferative cells. The dendritic cells obtained are infected with an adenoviral vector comprising a p53 gene and the p53 adenovirus-infected dendritic cells are administered to the patient. It is contemplated that infected dendritic cells will present self gene antigens to immune effector cells, stimulate an anti- self gene response in the patient and result in the destruction or lysis of hyperproliferative cells presenting mutant self gene antigen.

A. HYPERPROLIFERATIVE DISEASE

Cancer has become one of the leading causes of death in the Western world, second only behind heart disease. Current estimates project that one person in three in the U.S. will develop cancer, and that one person in five will die from cancer. Cancers can be viewed from an immunologic perspective as altered self cells, that have lost the normal growth-regulating mechanisms.

There are currently three major categories of oncogenes, reflecting their different activities. One category of oncogenes encode proteins that induce cellular proliferation. A second category of oncogenes, called tumor-suppressors genes or anti-oncogenes, function to inhibit excessive cellular proliferation. The third category of oncogenes, either block or induce apoptosis by encoding proteins that regulate programmed cell death.

In one embodiment of the present invention, the treatment of hyperproliferative disease involves the intradermal administration of a self gene expression construct to dendritic cells. It is contemplated that the dendritic cells present the processed self gene wild-type antigens to immune effector cells, which mount an anti-self gene response, resulting in the destruction or lysis of hyperproliferative cells presenting mutant self antigen. The three major categories of oncogenes are discussed below and listed in Table 1.

1. INDUCERS OF CELLULAR PROLIFERATION

The proteins that induce cellular proliferation further fall into various categories dependent on function. The commonality of all of these proteins is their ability to regulate cellular proliferation. For example, a form of PDGF, the sis oncogene is a secreted growth factor. Oncogenes rarely arise from genes encoding growth factors, and at the present, sis is the only known naturally occurring oncogenic growth factor.

The proteins fms, erbA, erbB and neu are growth factor receptors. Mutations to these receptors result in loss of regulatable function. For example, a point mutation affecting the transmembrane domain of the nue receptor protein results in the nue oncogene. The erbA oncogene is derived from the intracellular receptor for thyroid hormone. The modified oncogenic erbA receptor is believed to compete with the endogenous thyroid hormone receptor, causing uncontrolled growth.

The largest class of oncogenes are the signal transducing proteins (*e.g.*, src, abl and ras) are signal transducers. The protein src, is a cytoplasmic protein-tyrosine kinase, and its transformation from proto-oncogene to oncogene in some cases, results via mutations at tyrosine residue 527. In contrast, transformation of GTPase protein ras from proto-oncogene to oncogene, in one example, results from a valine to glycine mutation at amino acid 12 in the sequence, reducing ras GTPase activity.

The proteins jun, fos and myc are proteins that directly exert their effects on nuclear functions as transcription factors. Table 1 lists a variety of the oncogenes described in this section and many of those not described.

2. INHIBITORS OF CELLULAR PROLIFERATION

The tumor suppressor oncogenes function to inhibit excessive cellular proliferation. The inactivation of these genes results destroys their inhibitory activity, resulting in unregulated proliferation. The tumor suppressors p53, p16 and C-CAM are described below.

High levels of mutant p53 have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently-mutated gene in common human cancers. It is mutated in over 50% of human NSCLC (Hollstein *et al.*, 1991) and in a wide spectrum of other tumors. A variety of cancers have been associated with mutations of the p53 gene, which result in the loss of p53 tumor suppressor properties. Mutations in the p53 gene further account for approximately 50% of all cancers that develop (Vogelstein and Kinzler, 1992; Levine *et al.*, 1991), with the majority of these mutations being single-base missense mutations (Kovach *et al.*, 1996). It has been observed that mutations resulting in a loss of p53 function also result in high nuclear and cytoplasmic concentrations (*i.e.* overexpression) of mutant p53 protein (Oldstone *et al.*, 1992; Finlay *et al.*, 1988). In contrast, functional wild-type p53 protein is expressed at very low levels in cells.

The high cellular concentrations of p53 mutant protein has recently received much attention as an avenue for cancer immunotherapy. The general concept is to elicit an immune response against tumor cells presenting mutant p53 peptides bound to MHC molecules on the cell surface. The generation of an anti-tumor response using mutant p53 peptides as antigens has been demonstrated in several studies (McCarty *et al.*, 1998; Gabrilovich *et al.*, 1996; Mayordomo *et al.*, 1996; Zitvogel *et*

al., 1996) However, this approach to cancer immunotherapy has several limitations. For example, p53 mutations can occur at many different sites in the protein, making it necessary to identify the site of the mutation in each patient before creating a specific mutant peptide for p53 cancer therapy. Further, not all mutations are contained in regions of the protein known to bind to MHC molecules, and therefore would not elicit an anti-tumor response (DeLeo, 1998).

The limitations described above have stimulated the search for antigenic epitopes in wild-type p53 sequences common to the vast majority of tumor derived p53 proteins. Wild-type p53 peptide-specific cytotoxic T lymphocytes have been produced from human and murine responding lymphocytes, some of which recognized p53-overexpressing tumors *in vitro* and *in vivo* (Theobald, *et al.*, 1995; Ropke *et al.*, 1996; Nijman *et al.*, 1994; U.S. Patent 5,747,469, specifically incorporated herein by reference in its entirety). However, since the presentation of antigens is MHC class I restricted, only certain peptides can successfully be administered in certain patients, due to the polymorphic nature of the MHC class I peptide binding site. Further, it is not practical to identify all possible p53 peptides binding to a particular individuals repertoire of MHC molecules. Additionally, a peptide vaccine that does bind to a patient's class I MHC may not be sufficiently presented by MHC class II, the molecules crucial in the induction of $CD4^+$ T cell immune responses.

Researchers have to attempted to identify multiple p53 epitopes, which should permit more effective immune responses against tumor cells expressing multiple p53 genes with mutations at different sites. This could be accomplished by immunizing cells with intact wild-type p53 to take advantage of the overexpression of the whole p53 polypeptide in most human tumors. The dendritic cell (DC) is the cell type best suited for vaccine antigen delivery (described further in section B), as they are the most potent antigen presenting cells, effective in the stimulation of both primary and secondary immune responses (Steinman, 1991; Celluzzi and Falo, 1997). It is contemplated in the present invention that the transduction of dendritic cells with

wild-type p53 protein, using a viral expression construct, will elicit a potent antitumor immune response specific for a variety of cells expressing different mutant p53 proteins. Further, since most mutations of p53 are single-base missense mutations, the approach of the present invention overcomes the limitations of identifying the site of the p53 mutation and subsequent preparation of a customized mutant peptide for immunotherapy. Thus, the method of the present invention provides the basis for a simple and novel approach to immunotherapy based cancer treatment.

Wild-type p53 is recognized as an important growth regulator in many cell types. Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53. Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Another inhibitor of cellular proliferation is p16. The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G_1. The activity of this enzyme may be to phosphorylate Rb at late G_1. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, the $p16^{INK4}$ has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano *et al.*, 1993; Serrano *et al.*, 1995). Since the $p16^{INK4}$ protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

$p16^{INK4}$ belongs to a newly described class of CDK-inhibitory proteins that also includes $p16^{B}$, $p21^{WAF1}$, and $p27^{KIP1}$. The $p16^{INK4}$ gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the $p16^{INK4}$ gene are frequent in human tumor cell lines. This evidence suggests that the $p16^{INK4}$ gene is a tumor suppressor gene. This interpretation has been challenged, however, by the observation that the frequency of the $p16^{INK4}$ gene alterations is much lower in primary uncultured tumors than in cultured cell lines (Caldas *et al.*, 1994; Cheng *et al.*, 1994; Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Kamb *et al.*, 1994; Mori *et al.*, 1994; Okamoto *et al.*, 1994; Nobori *et al.*, 1995; Orlow *et al.*, 1994; Arap *et al.*, 1995). Restoration of wild-type $p16^{INK4}$ function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto, 1994; Arap, 1995).

C-CAM is expressed in virtually all epithelial cells (Odin and Obrink, 1987). C-CAM, with an apparent molecular weight of 105 kD, was originally isolated from the plasma membrane of the rat hepatocyte by its reaction with specific antibodies that neutralize cell aggregation (Obrink, 1991). Recent studies indicate that, structurally, C-CAM belongs to the immunoglobulin (Ig) superfamily and its sequence is highly homologous to carcinoembryonic antigen (CEA) (Lin and Guidotti, 1989). Using a baculovirus expression system, Cheung *et al.* (1993) demonstrated that the first Ig domain of C-CAM is critical for cell adhesive activity.

Cell adhesion molecules, or CAM's are known to be involved in a complex network of molecular interactions that regulate organ development and cell differentiation (Edelman, 1985). Recent data indicate that aberrant expression of CAM's maybe involved in the tumorigenesis of several neoplasms; for example, decreased expression of E-cadherin, which is predominantly expressed in epithelial cells, is associated with the progression of several kinds of neoplasms (Edelman and Crossin, 1991; Frixen *et al.*, 1991; Bussemakers *et al.*, 1992; Matsura *et al.*, 1992; Umbas *et al.*, 1992). Also, Giancotti and Ruoslahti (1990) demonstrated that increasing expression of $\alpha_5\beta_1$ integrin by gene transfer can reduce tumorigenicity of

Chinese hamster ovary cells *in vivo*. C-CAM now has been shown to suppress tumors growth *in vitro* and *in vivo*.

Other tumor suppressors that may be employed according to the present invention include RB, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, zac1, p73, VHL, MMAC1, FCC and MCC (see Table 1).

3. REGULATORS OF PROGRAMMED CELL DEATH

Apoptosis, or programmed cell death, is an essential occurring process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing carcinogenesis (Kerr *et al.*, 1972). The Bcl-2 family of proteins and ICE-like proteases have been demonstrated to be important regulators and effectors of apoptosis in other systems. The Bcl-2 protein, discovered in association with follicular lymphoma, plays a prominent role in controlling apoptosis and enhancing cell survival in response to diverse apoptotic stimuli (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Cleary *et al.*, 1986; Tsujimoto *et al.*, 1985; Tsujimoto and Croce, 1986). The evolutionarily conserved Bcl-2 protein now is recognized to be a member of a family of related proteins which can be categorized as death agonists or death antagonists.

Subsequent to its discovery, it was shown that Bcl-2 acts to suppress cell death triggered by a variety of stimuli. Also, it now is apparent that there is a family of Bcl-2 cell death regulatory proteins which share in common structural and sequence homologies. These different family members have been shown to either possess similar functions to Bcl-2 (*e.g.*, Bcl_{XL}, Bcl_W, Mcl-1, A1, Bfl-1) or counteract Bcl-2 function and promote cell death (*e.g.*, Bax, Bak, Bik, Bim, Bid, Bad, Harakiri).

TABLE 1

ONCOGENES

Gene	*Source*	*Human Disease*	*Function*
Growth Factors[1]			FGF family member
HST/KS	Transfection		
INT-2	MMTV promoter insertion		FGF family member
INTI/WNTI	MMTV promoter insertion		Factor-like
SIS	Simian sarcoma virus		PDGF B
Receptor Tyrosine Kinases[1,2]			
ERBB/HER	Avian erythroblastosis virus; ALV promoter insertion; amplified human tumors	Amplified, deleted squamous cell cancer; glioblastoma	EGF/TGF-α/ amphiregulin/ hetacellulin receptor
ERBB-2/NEU/HER-2	Transfected from rat glioblatoms	Amplified breast, ovarian, gastric cancers	Regulated by NDF/ heregulin and EGF-related factors
FMS	SM feline sarcoma virus		CSF-1 receptor
KIT	HZ feline sarcoma virus		MGF/Steel receptor hematopoieis
TRK	Transfection from human colon cancer		NGF (nerve growth factor) receptor
MET	Transfection from human osteosarcoma		Scatter factor/HGF receptor
RET	Translocations and point mutations	Sporadic thyroid cancer; familial medullary thyroid cancer; multiple endocrine neoplasias 2A and 2B	Orphan receptor Tyr kinase

TABLE 1 (CONT'D)

ROS	URII avian sarcoma virus		Orphan receptor Tyr kinase
PDGF receptor	Translocation	Chronic myclomonocytic leukemia	TEL(ETS-like transcription factor)/ PDGF receptor gene fusion
TGF-β receptor		Colon carcinoma mismatch mutation target	
NONRECEPTOR TYROSINE KINASES[1]			
ABI.	Abelson Mul.V	Chronic myelogenous leukemia translocation with BCR	Interact with RB, RNA polymerase, CRK, CBL
FPS/FES	Avian Fujinami SV;GA FeSV		
LCK	Mul.V (murine leukemia virus) promoter insertion		Src family; T cell signaling; interacts CD4/CD8 T cells
SRC	Avian Rous sarcoma virus		Membrane-associated Tyr kinase with signaling function; activated by receptor kinases
YES	Avian Y73 virus		Src family; signaling
SER/THR PROTEIN KINASES[1]			
AKT	AKT8 murine retrovirus		Regulated by PI(3)K?; regulate 70-kd S6 k?
MOS	Maloney murine SV		GVBD; cystostatic factor; MAP kinase kinase

TABLE 1 (CONT'D)

PIM-1	Promoter insertion mouse		
RAF/MIL	3611 murine SV; MH2 avian SV		Signaling in RAS pathway
MISCELLANEOUS CELL SURFACE[1]			
APC	Tumor suppressor	Colon cancer	Interacts with catenins
DCC	Tumor suppressor	Colon cancer	CAM domains
E-cadherin	Candidate tumor suppressor	Breast cancer	Extracellular homotypic binding; intracellular interacts with catenins
PTC/NBCCS	Tumor suppressor and *Drosophilia* homology	Nevoid basal cell cancer syndrome (Gorline syndrome)	12 transmembrane domain; signals through Gli homogue CI to antagonize hedgehog pathway
TAN-1 Notch homologue	Translocation	T-ALI.	Signaling?
MISCELLANEOUS SIGNALING[1,3]			
BCL-2	Translocation	B-cell lymphoma	Apoptosis
CBL	Mu Cas NS-1 V		Tyrosine-phosphorylated RING finger interact Abl
CRK	CT1010 ASV		Adapted SH2/SH3 interact Abl
DPC4	Tumor suppressor	Pancreatic cancer	TGF-β-related signaling pathway
MAS	Transfection and tumorigenicity		Possible angiotensin receptor
NCK			Adaptor SH2/SH3
GUANINE NUCLEOTIDE EXCHANGERS AND BINDING PROTEINS[3,4]			
BCR		Translocated with ABL in CML	Exchanger; protein kinase
DBL	Transfection		Exchanger

TABLE 1 (CONT'D)

GSP			
NF-1	Hereditary tumor suppressor	Tumor suppressor neurofibromatosis	RAS GAP
OST	Transfection		Exchanger
Harvey-Kirsten, N-*RAS*	HaRat SV; Ki RaSV; Balb-MoMuSV; transfection	Point mutations in many human tumors	Signal cascade
VAV	Transfection		S112/S113; exchanger
NUCLEAR PROTEINS AND TRANSCRIPTION FACTORS[1,5-9]			
BRCA1	Heritable suppressor	Mammary cancer/ovarian cancer	Localization unsettled
BRCA2	Heritable suppressor	Mammary cancer	Function unknown
ERBA	Avian erythroblastosis virus		thyroid hormone receptor (transcription)
ETS	Avian E26 virus		DNA binding
EVI1	MuLV promotor insertion	AML	Transcription factor
FOS	FBI/FBR murine osteosarcoma viruses		1 transcription factor with c-JUN
GLI	Amplified glioma	Glioma	Zinc finger; cubitus interruptus homologue is in hedgehog signaling pathway; inhibitory link PTC and hedgehog
HMGG/LIM	Translocation *t*(3:12) *t*(12:15)	Lipoma	Gene fusions high mobility group HMGI-C (XT-hook) and transcription factor LIM or acidic domain
JUN	ASV-17		Transcription factor AP-1 with FOS

TABLE 1 (CONT'D)

MLL/VHRX + ELI/MEN	Translocation/fusion ELL with MLL trithorax-like gene	Acute myeloid leukemia	Gene fusion of DNA-binding and methyl transferase MLL with ELI RNA pol II elongation factor
MYB	Avian myeloblastosis virus		DNA binding
MYC	Avian MC29; translocation B-cell lymphomas; promoter insertion avian leukosis virus	Burkitt's lymphoma	DNA binding with MAX partner; cyclin regulation; interact RB?; regulate apoptosis?
N-MYC	Amplified	Neuroblastoma	
L-MYC		Lung cancer	
REL	Avian retriculoendotheliosis virus		NF-κB family transcription factor
SKI	Avian SKV770 retrovirus		Transcription factor
VHL	Heritable suppressor	Von Hippel-Landau syndrome	Negative regulator or elongin; transcriptional elongation complex
WT-1		Wilm's tumor	Transcription factor
CELL CYCLE/DNA DAMAGE RESPONSE[10-21]			
ATM	Hereditary disorder	Ataxia-telangiectasia	Protein/lipid kinase homology; DNA damage response upstream in P53 pathway
BCL-2	Translocation	Follicular lymphoma	Apoptosis
FACC	Point mutation	Fanconi's anemia group C (predisposition leukemia	

TABLE 1 (CONT'D)

FHIT	Fragile site 3p14.2	Lung carcinoma	Histidine triad-related diadenosine 5',3''''-P^1.p^4 tetraphosphate asymmetric hydrolase
hMLI/MutL		HNPCC	Mismatch repair; MutL homologue
hMSH2/MutS		HNPCC	Mismatch repair; MutS homologue
hPMS1		HNPCC	Mismatch repair; MutL homologue
hPMS2		HNPCC	Mismatch repair; MutL homologue
INK4/MTS1	Adjacent INK-4B at 9p21; CDK complexes	Candidate MTS1 suppressor and MLM melanoma gene	p16 CDK inhibitor
INK4B/MTS2		Candidate suppressor	p15 CDK inhibitor
MDM-2	Amplified	Sarcoma	Negative regulator p53
p53	Association with SV40 T antigen	Mutated >50% human tumors, including hereditary Li-Fraumeni syndrome	Transcription factor; checkpoint control; apoptosis
PRAD1/BCL1	Translocation with parathyroid hormone or IgG	Parathyroid adenoma; B-CLL	Cyclin D
RB	Hereditary retinoblastoma; association with many DNA virus tumor antigens	Retinoblastoma; osteosarcoma; breast cancer; other sporadic cancers	Interact cyclin/cdk; regulate E2F transcription factor
XPA		xeroderma pigmentosum; skin cancer predisposition	Excision repair; photo-product recognition; zinc finger

4. NON-CANCER HYPERPROLIFERATIVE DISEASES

In one embodiment of the present invention, it is contemplated that non-cancer hyperproliferative diseases may treated by administering a self gene expression construct capable of eliciting an anti-self gene response. Some of the hyperproliferative diseases contemplated for treatment in the present invention are psoriasis, rheumatoid arthritis (RA), inflamatory bowel disease (IBD), osteo arthritis (OA) and pre-neoplastic lesions in the lung.

B. PATHOGEN INDUCED DISEASE

In other embodiments of the present invention, a method for treating a pathogen-induced disease in a subject in which pathogen-induced disease is characterized by an alteration or increased expression of a pathogen gene product in at least some of the pathogen-induced cells is contemplated. Following identification of altered or increased expression of a pathogen gene product, an expression construct comprising a pathogen gene under the control a promoter operable in eukaryotic cells is intradermally administering to the subject. It is contemplated that the pathogen gene product expressed in the dendritic cells is presented to immune effector cells, stimulating an anti-pathogen gene product response.

In another embodiment, it is contemplated that pathogen such as bacterium, virus, fungus, parasitic worm, amoebae and mycoplasma can be treated using the method of the present invention. For example, anti-pathogen responses to bacteria such as richettsia, listeria and histolytica, viri such as HIV, HBV, HCV, HSV, HPV, EBV and CMV, and fungi such as hitoplasma, coccidis, immitis, aspargillus, actinomyces, blastomyces, candidia and streptomyces, are contemplated in the present invention.

C. IMMUNOLOGIC RESPONSES RELATED TO SELF GENE TUMOROGENICITY

In one embodiment of the present invention, hyperproliferative disease in which p53 expression is upregulated in the hyperproliferative cells is treated by

administering a p53 expression construct capable of eliciting an anti-p53 response. Following delivery of the p53 expression construct to a given antigen presenting cell, a cascade of immunologic events must ensue to stimulate the desired anti-p53 response. Thus, a basic understanding of the immunologic responses related to p53 expressionand more generally, self gene expression in hyperproliferative disease is necessary.

1. CYTOTOXIC T LYMPHOCYTES

T lymphocytes arise from hematopoietic stem cells in the bone marrow, and migrate to the thymus gland to mature. T cells express a unique antigen binding receptor on their membrane (T-cell receptor), which can only recognize antigen in association with major histocompatibility complex (MHC) molecules on the surface of other cells. There are at least two populations of T cells, known as T helper cells and T cytotoxic cells. T helper cells and T cytotoxic cells are primarily distinguished by their display of the membrane bound glycoproteins CD4 and CD8, respectively. T helper cells secret various lymphokines, that are crucial for the activation of B cells, T cytotoxic cells, macrophages and other cells of the immune system. In contrast, a T cytotoxic cells that recognizes an antigen-MHC complex proliferates and differentiates into an effector cell called a cytotoxic T lymphocyte (CTL). CTLs eliminate cells of the body displaying antigen, such as virus infected cells and tumor cells, by producing substances that result in cell lysis.

An important aspect of the present invention is the stimulation of a CTL response directed against wild-type self gene antigen. It has been observed that mutations of the p53 gene result in the overexpression of the mutant p53 protein in tumor cells (Harris, 1996), while wild-type p53 is expressed at low levels in normal cells. It has further been demonstrated that wild-type and mutant p53 peptides can stimulate a CTL response against tumor cells expressing p53 antigenic peptides (DeLeo, 1998; Mayordomo *et al.*, 1996). It is contemplated in the present invention that a similar anti- self gene CTL response will be stimulated by immunizing dendritic

cells with intact wild-type self gene polypeptide, and thus can be used as a treatment for hyperproliferative disease.

2. ANTIGEN-PRESENTING CELLS

Antigen-presenting cells, which include macrophages, B lymphocytes, and dendritic cells, are distinguished by their expression of a particular MHC molecule. APCs internalize antigen and re-express a part of that antigen, together with the MHC molecule on their outer cell membrane.

In a preferred embodiment of the present invention, dendritic cells are the antigen-presenting cells of choice for self gene delivery and antigen presentation. Dendritic cells are the most potent antigen-presenting cells for the initiation of antigen-specific T cell activation (Arthur *et al.*, 1997). They are also excellent candidates for short term culture and a variety of gene transfer methods (*e.g.*, DNA/liposome complexes, electroporation, CaPO4 precipitation, and recombinant adenovirus) (Arthur *et al.*, 1997). Human and mouse dendritic cells have been successfully modified by adenoviral gene transfer (Sonderbye *et al.*, 1998). In this study, an adenovirus (AdLacZ) was used to express intracellular beta-galactosidase (beta-gal) antigen in the dendritic cells, with approximately 40% of the cells transduced with AdLacZ expressing high levels of beta-gal. In addition, the subcutaneous immunization of mouse dendritic cells with the ovalbumin (OVA) peptide induced an OVA-specific CD8+ CTL response (Celluzzi and Falo, 1997).

3. MAJOR HISTOCOMPATIBILITY COMPLEX

The major histocompatibility complex (MHC) is a large genetic complex with multiple loci. The MHC loci encode two major classes of MHC membrane molecules, referred to as class I and class II MHCs. T helper lymphocytes generally recognize antigen associated with MHC class II molecules, and T cytotoxic lymphocytes recognize antigen associated with MHC class I molecules. In humans the MHC is refereed to as the HLA complex and in mice the H-2 complex. An important aspect of the present invention is the immunization of dendritic cells with

the intact wild-type self gene to take advantage of the relative overexpression of the whole self gene molecule in most human tumors. The approach of p53 immunotherapy is contemplated in one embodiment, to overcome previous immunotherapies that immunized animals with mutant p53 peptides as antigens (Gabrilovich *et al.*, 1996; Mayordomo *et al.*, 1996; Zitgovel *et al.*, 1996). Although the approaches above using mutant p53 peptides were effective at generating anti-tumor responses, they have several limitations. For example, p53 mutations and other self genes occur at many sites in the protein, making it necessary to identify the site of mutation in each patient before constructing a customized mutant peptide for therapy. Furthermore, not all mutations are contained in regions of the protein known to bind to MHC molecules. In another study using wild-type 53 peptides, CTLs were generated from human and murine responding lymphocytes, some of which recognized p53 overexpressing tumors *in vitro* (Theobald *et al.*, 1995; Ropke *et al.*, 1996; Nijman *et al.*, 1994). However, since presentation of antigens is MHC class I restricted, only certain oligopeptides can be used in certain patients, because of the highly polymorphic MHC class I peptide binding site. It is contemplated in the present invention that immunizing dendritic cells with intact, wild-type self gene protein, will generate a variety of self gene antigens for MHC class I presentation and thus effectively stimulate a cytolytic T lymphocyte response.

D. Assays for Self Gene Upregulation or Altered Expression

In one embodiment of the present invention, the identification of a patient with a hyperproliferative disease in which self gene expression is upregulated is desired. In patients with a detected hyperproliferative disease, a sample of the hyperproliferative tissue will be used to assay upregulation. A wide variety of detection methods can be employed in the present invention to detect the self gene status of a cell. There are numerous antibodies to the oncogenic proteins, hence any assay that utilizes antibodies for detection, for example, ELISAs, Western Blotting, immunoassay techniques, *etc.* are contemplated as useful in the present invention. Alternatively, assays that employ nucleotide probes may be used to identify the presence of self gene, for example, Southern blotting, Northern blotting or PCR™ techniques. All the

above techniques are well known to one of skill in the art and could be utilized in the present invention without undue experimentation.

1. ELISAs, IMMUNOASSAY AND IMMUNOHISTOLOGICAL ASSAY.

In a preferred embodiment of the present invention, immunohistological assays are used to detect self gene increased or altered expression in tumor samples (*e.g.*, tissue sections). Exemplary methods of immunohistochemistry assays and immunfluorescence assays have previously been described (U.S. Patent 5,858,723; WO94/11514, specifically incorporated herein by reference in its entirety). Further immunoassays encompassed by the present invention include, but are not limited to those described in U.S. Patent 4,367,110 (double monoclonal antibody sandwich assay) and U.S. Patent 4,452,901 (western blot). Other assays include immunoprecipitation of labeled ligands and immunocytochemistry, both *in vitro* and *in vivo*. Immunoassays generally are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art.

In one exemplary ELISA, the anti- self gene antibodies are immobilized on a selected surface, such as a well in a polystyrene microtiter plate, dipstick or column support. Then, a test composition suspected of containing the desired antigen, such as a clinical sample, is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound antigen may be detected. Detection is generally achieved by the addition of another antibody, specific for the desired antigen, that is linked to a detectable label. This type of ELISA is known as a "sandwich ELISA." Detection also may be achieved by the addition of a second antibody specific for the desired antigen, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

2. SOUTHERN AND NORTHERN BLOTTING TECHNIQUES

Southern and Northern blotting are commonly used techniques in molecular biology and well within the grasp of one skilled in the art. Southern and Northern blotting samples are obtained from the hyperproliferative tissue. The DNA and RNA from test cells is recovered by gentle cell rupture in the presence of a cation chelator such as EDTA. The proteins and other cell milieu are removed by admixing with saturated phenol or phenol/chloroform and centrifugation of the emulsion. The DNA and RNA is in the upper aqueous phase, it is deproteinised and mixed with ethanol. This solution allows the DNA and RNA to precipitate, the DNA and RNA can then be recover using centrifugation. In the case of RNA extraction, RNAse inhibitors such as DEPC are needed to prevent RNA degradation.

Electrophoresis in agarose or polyacrylamide gels is the most usual way to separate DNA and RNA molecules. Southern blotting will confirm the identity of the self gene encoding DNA. This is achieved by transferring the DNA from the intact gel onto nitrocellulose paper. The nitrocellulose paper is then washed in buffer that has for example, a radiolabelled cDNA containing a sequence complementary to wild-type self gene DNA. The probe binds specifically to the DNA that encodes a region of self gene and can be detected using autoradiography by contacting the probed nitrocellulose paper with photographic film. Self gene -encoding mRNA can be detected in a similar manner by a process known as Northern blotting. For a more detailed description of buffers gel preparation, electrophoresis condition *etc.*, the skilled artisan is referred to Sambrook, 1989.

3. POLYMERASE CHAIN REACTION (PCR™)

PCR™ is a powerful tool in modern analytical biology. Short oligonucleotide sequences usually 15-35 bp in length are designed, homologous to flanking regions either side of the self gene sequences to be amplified. The primers are added in excess to the source DNA, in the presence of buffer, enzyme, and free nucleotides. The source DNA is denatured at 95°C and then cooled to 50-60°C to allow the

primers to anneal. The temperature is adjusted to the optimal temperature for the polymerase for an extension phase. This cycle is repeated 25-40 times.

In particular the present invention uses PCR™ to detect the self gene status of cells. Mutations in the self gene are first detected with Single Strand Conformation Polymorphism (SSCP) which is based on the electrophoretic determination of conformational changes in single stranded DNA molecules induced by point mutations or other forms of slight nucleotide changes. To identify where the mutation is located at within the self gene, each exon is separately amplified by PCR™ using primers specific for the particular exon. After amplification, the PCR™ product is denatured and separated out on a polyacrylamide gel to detect a shift in mobility due to a conformational change which resulted because of a point mutation or other small nucleotide change in the gene. Mutations result in a change in the physical conformation of the DNA as well as change in the electrical charge of the molecule. Thus during electrophoresis when an electrical charge is applied to the molecule, DNA that is slightly different in shape and charge as compared to wild-type will move at a different rate and thus occupy a different position in the gel.

After determination of which DNA fragment contains the mutation, the specific nucleotide changes are detected by DNA sequencing of the amplified PCR™ product. Sequencing of linear DNA breaks down the DNA molecule into its individual nucleotides in the order with which they are assembled in the intact molecule. Separation of the individual nucleotides by electrophoresis on a sequencing gel allows detection of individual nucleotide changes compared to wild-type and is used to determine homo- or heterozygocity of a mutation, which is easily distinguished by the appearance of a single or double band in the sequencing gel.

E. SELF GENE DELIVERY

Many types of cancer have been associated with mutations in oncogenes. These mutations typically result in the overexpression of a mutant self gene protein in tumor cells. It has been further demonstrated that wild-type p53 peptide specific

cytotoxic T lymphocytes were generated from human and murine responding lymphocytes and recognized p53 overexpressing tumors *in vitro* (Theobald *et al.*, 1995; Ropke *et al.*, 1996; Nijman *et al.*, 1994). The present invention contemplates the *in vivo* treatment of hyperproliferative diseases by eliciting an anti-self gene immune response directed against cells presenting self gene antigen on their surface. In certain embodiments of the present invention, an expression construct comprising a self gene under the control of a promoter operable in eukaryotic cells is administered and expressed in dendritic cells in order to prime an immune response against p53.

1. VIRAL TRANSFORMATION

a. ADENOVIRAL INFECTION

One method for delivery of the recombinant DNA involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a recombinant gene construct that has been cloned therein.

The vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization or adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats

(ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the

vector. More than 80% of the adenovirus viral genome remains in the vector backbone.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.* (1995) have disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

The adenovirus vector may be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known,

and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.* (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Animal studies have suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

b. RETROVIRAL INFECTION

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact sequence from the recombinant virus inserts

upstream from the gag, pol, env sequence integrated in the host cell genome. However, packaging cell lines are available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

c. AAV INFECTION

Adeno-associated virus (AAV) is an attractive vector system for use in the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue culture (Muzyczka, 1992). AAV has a broad host range for infectivity (Tratschin, *et al.*, 1984; Laughlin, *et al.*, 1986; Lebkowski, *et al.*, 1988; McLaughlin, *et al.*, 1988), which means it is applicable for use with the present invention. Details concerning the generation and use of rAAV vectors are described in U.S. Patent No. 5,139,941 and U.S. Patent No. 4,797,368, each incorporated herein by reference.

Studies demonstrating the use of AAV in gene delivery include LaFace *et al.* (1988); Zhou *et al.* (1993); Flotte *et al.* (1993); and Walsh *et al.* (1994). Recombinant AAV vectors have been used successfully for *in vitro* and *in vivo* transduction of marker genes (Kaplitt *et al.*, 1994; Lebkowski *et al.*, 1988; Samulski *et al.*, 1989; Shelling and Smith, 1994; Yoder *et al.*, 1994; Zhou *et al.*, 1994; Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1985; McLaughlin *et al.*, 1988) and genes involved in human diseases (Flotte *et al.*, 1992; Luo *et al.*, 1994; Ohi *et al.*, 1990; Walsh *et al.*, 1994; Wei *et al.*, 1994). Recently, an AAV vector has been approved for phase I human trials for the treatment of cystic fibrosis.

AAV is a dependent parvovirus in that it requires coinfection with another virus (either adenovirus or a member of the herpes virus family) to undergo a productive infection in cultured cells (Muzyczka, 1992). In the absence of coinfection with helper virus, the wild-type AAV genome integrates through its ends into human chromosome 19 where it resides in a latent state as a provirus (Kotin *et al.*, 1990; Samulski *et al.*, 1991). rAAV, however, is not restricted to chromosome 19 for integration unless the AAV Rep protein is also expressed (Shelling and Smith, 1994).

When a cell carrying an AAV provirus is superinfected with a helper virus, the AAV genome is "rescued" from the chromosome or from a recombinant plasmid, and a normal productive infection is established (Samulski *et al.*, 1989; McLaughlin *et al.*, 1988; Kotin *et al.*, 1990; Muzyczka, 1992).

Typically, recombinant AAV (rAAV) virus is made by cotransfecting a plasmid containing the gene of interest flanked by the two AAV terminal repeats (McLaughlin *et al.*, 1988; Samulski *et al.*, 1989; each incorporated herein by reference) and an expression plasmid containing the wild-type AAV coding sequences without the terminal repeats, for example pIM45 (McCarty *et al.*, 1991; incorporated herein by reference). The cells are also infected or transfected with adenovirus or plasmids carrying the adenovirus genes required for AAV helper function. rAAV virus stocks made in such fashion are contaminated with adenovirus which must be physically separated from the rAAV particles (for example, by cesium chloride density centrifugation). Alternatively, adenovirus vectors containing the AAV coding regions or cell lines containing the AAV coding regions and some or all of the adenovirus helper genes could be used (Yang *et al.*, 1994a; Clark *et al.*, 1995). Cell lines carrying the rAAV DNA as an integrated provirus can also be used (Flotte *et al.*, 1995).

d. Other Viral Vectors

Other viral vectors may be employed as constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990). Alternatively, Alphavirus vectors and replicons may be employed (Leitner *et al.*, 2000; Caley *et al.*, 1999).

A molecularly cloned strain of Venezuelan equine encephalitis (VEE) virus has been genetically refined as a replication competent vaccine vector for the

expression of heterologous viral proteins (Davis *et al.*, 1996). Studies have demonstrated that VEE infection stimulates potent CTL responses and has been sugested that VEE may be an extremely useful vector for immunizations (Caley *et al.*, 1997). It is contemplated in the present invention, that VEE virus may be useful in targeting dendritic cells.

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

In still further embodiments of the present invention, the nucleic acids to be delivered are housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate receptors of the target cell and deliver the contents to the cell. A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification can permit the specific infection of hepatocytes via sialoglycoprotein receptors.

Another approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin

components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

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2. NON-VIRAL DELIVERY

In addition to viral delivery of the self gene, the following are additional methods of recombinant gene delivery to a given host cell and are thus considered in the present invention.

10

a. ELECTROPORATION

In certain preferred embodiments of the present invention, the gene construct is introduced into the dendritic cells via electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge.

15

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter *et al.*, 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa *et al.*, 1986) in this
20 manner.

It is contemplated that electroporation conditions for dendritic cells from different sources may be optimized. One may particularly wish to optimize such parameters as the voltage, the capacitance, the time and the electroporation media
25 composition. The execution of other routine adjustments will be known to those of skill in the art.

b. PARTICLE BOMBARDMENT

Another embodiment of the invention for transferring a naked DNA construct
30 into cells involves particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce

cell membranes and enter cells without killing them (Klein *et al.*, 1987). The microprojectiles used have consisted of biologically inert substances such as tungsten, platinum or gold beads.

It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using particle bombardment. It is contemplated that particles may contain DNA rather than be coated with DNA. Hence it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). Another method involves the use of a Biolistic Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as stainless steel or Nytex screen, onto a filter surface covered with cells in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectile aggregates and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters, or alternatively on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded.

In bombardment transformation, one may optimize the prebombardment culturing conditions and the bombardment parameters to yield the maximum numbers

of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity or either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of primordial germ cells.

Accordingly, it is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance and helium pressure. One may also optimize the trauma reduction factors by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art.

c. Calcium Phosphate Co-Precipitation or DEAE-Dextran Treatment

In other embodiments of the present invention, the transgenic construct is introduced to the cells using calcium phosphate co-precipitation. Mouse primordial germ cells have been transfected with the SV40 large T antigen, with excellent results (Watanabe *et al.*, 1997). Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.*, 1990).

In another embodiment, the expression construct is delivered into the cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

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d. Direct Microinjection or Sonication Loading

Further embodiments of the present invention include the introduction of the gene construct by direct microinjection or sonication loading. Direct microinjection has been used to introduce nucleic acid constructs into *Xenopus* oocytes (Harland and
10 Weintraub, 1985), and LTK$^-$ fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer *et al.*, 1987).

e. Liposome Mediated Transformation

In a further embodiment of the invention, the gene construct may be entrapped
15 in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water
20 and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated is a gene construct complexed with Lipofectamine (Gibco BRL) or DOTAP-Cholesterol formulations.

Liposome-mediated nucleic acid delivery and expression of foreign DNA
25 *in vitro* has been very successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells.

30 In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell

membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1.

3. VECTORS AND REGULATORY SIGNALS

Vectors of the present invention are designed, primarily, to transform dendritic cells with the self gene under the control of regulated eukaryotic promoters (*i.e.*, inducible, repressable, tissue specific). Also, the vectors usually will contain a selectable marker if, for no other reason, to facilitate their production *in vitro*. However, selectable markers may play an important role in producing recombinant cells and thus a discussion of promoters is useful here. Table 2 and Table 3 below, list inducible promoter elements and enhancer elements, respectively.

Preferred for use in the present invention is the cytomegalovirus (CMV) promoter. This promoter is commercially available from Invitrogen in the vector pcDNAIII, which is preferred for use in the present invention. Also contemplated as useful in the present invention are the dectin-1 and dectin-2 promoters. Below are a list of additional viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the present invention. Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of structural genes encoding oligosaccharide processing enzymes, protein folding accessory proteins, selectable marker proteins or a heterologous protein of interest.

Table 2 Table 1 - Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987; Karin ®, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Fonta *et al.*, 1985; Sakai *et al.*, 1986
ß-Interferon	poly(rI)X poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angle *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angle *et al.*, 1987b
SV40	Phorbol Ester (TFA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a,b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

Table 3 - Other Promoter/Enhancer Elements

Promoter/Enhance	References
Immunoglobulin Heavy Chain	Hanerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1988; Kiledjian *et al.*, 1988; Porton *et al.*, 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987, Winoto and Baltimore, 1989; Redondo *et al.*, 1990
HLA DQ α and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1985
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-DRα	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*, 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989a
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Omitz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin Gene	Pinkert *et al.*, 1987, Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
e-fos	Cohen *et al.*, 1987

Table 3 – (CONT'D)

c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsch *et al.*, 1990
α-1- antitrypsin	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987 Schaffner *et al.*, 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; deVilliers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988

Table 3 – (CONT'D)

Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a,b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987, Stephens and Hentschel, 1987; Glue *et al.*, 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rowen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989
Dectin-1	
Dectin-2	

The use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′–methylated cap-dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open

reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message. Another signal that may prove useful is a polyadenylation signal (hGH, BGH, SV40).

As discussed above, in certain embodiments of the invention, a cell may be identified and selected *in vitro* or *in vivo* by including a marker in the expression construct. Such markers confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually, the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin, tetracycline and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed.

The promoters and enhancers that control the transcription of protein encoding genes in eukaryotic cells are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV 40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these

specificities. Aside from this operational distinction, enhancers and promoters are very similar entities.

Promoters and enhancers have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

In any event, it will be understood that promoters are DNA elements which when positioned functionally upstream of a gene leads to the expression of that gene. Most transgene constructs of the present invention are functionally positioned downstream of a promoter element.

F. PHARMACEUTICAL COMPOSITIONS AND ROUTES OF SELF GENE DELIVERY

In a preferred embodiment of the present invention, a method of treating a subject with a hyperproliferative disease in which self gene expression is increased or altered is contemplated. Hyperproliferative diseases that are most likely to be treated in the present invention are those that result from mutations in the self gene and the overexpression of self gene protein in the hyperproliferative cells. Examples of hyperproliferative diseases contemplated for treatment are lung cancer, head and neck cancer, breast cancer, pancreatic cancer, prostate cancer, renal cancer, bone cancer, testicular cancer, cervical cancer, gastrointestinal cancer, lymphomas, pre-neoplastic lesions in the lung, colon, breast and -bladder and any other hyperproliferative diseases that involve mutations and upregulation of self gene expression. An important aspect of this embodiment is the delivery of a self gene adenoviral vector to dendritic cells, for processing and presentation of self gene antigenic peptides to immune effector cells, thereby stimulating an anti- self gene response. In one embodiment, a self gene adenovirus concentration range of 100-300 PFU/cell transduces greater than 50% of the dendritic cells. The preferred mode of delivering the self gene construct in the present invention is by adenoviral vector.

In a preferred embodiment of the present invention, a method of treating a subject with a hyperproliferative disease in which p53 expression is upregulated is contemplated. Hyperproliferative diseases that are most likely to be treated in the present invention are those that result from mutations in the p53 gene and the overexpression of p53 protein in the hyperproliferative cells. Examples of hyperproliferative diseases contemplated for treatment are lung cancer, head and neck cancer, breast cancer, pancreatic cancer, prostate cancer, renal cancer, bone cancer, testicular cancer, cervical cancer, gastrointestinal cancer, lymphomas, pre-neoplastic lesions in the lung, colon, rectal, breast and -bladder and any other hyperproliferative diseases that involve mutations and upregulation of p53 expression. An important aspect of this embodiment is the delivery of a p53 adenoviral vector to dendritic cells, for processing and presentation of p53 antigenic peptides to immune effector cells, thereby stimulating an anti-p53 response. In one embodiment, a p53 adenovirus

concentration range of 100-300 PFU/cell transduces greater than 50% of the dendritic cells. The preferred mode of delivering the p53 adenoviral vector construct in the present invention is by intradermal injection of dendritic cells. In certain embodiments, the injection site is pretreated with chemokines or cytokines to elicit dendritic cell migration and maturation to the site of intradermal injection. In further embodiments, administration of the self gene adenoviral vector to dendritic cells comprises multiple intradermal injections. For example, the treatment of certain cancer types may require at least 3 or more immunizations, every 2-4 weeks. Dendritic cell intradermal injection may further be performed local, regional, or distal to the site of tumor growth, as well as subqutaneous, intraperitoneal or injection into or near a draining lymph node. Identifying, isolating, and obtaining dendritic cells are described below, in section H.

In certain embodiments, the present invention also concerns formulations of one or more self gene adenovirus compositions for administration to a mammal, that transduces dendritic cells of the mammal. For the treatment of hyperproliferative disease in humans, it is contemplated that the adenovirus vector is replication-defective, comprising a self gene under the control of a promoter operable in eukaryotic cells (*e.g.*, CMV IE, dectin-1, dectin-2). It will also be understood that, if desired, the self gene compositions disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, various pharmaceutically-active agents. As long as the composition comprises at least one self gene expression construct, there is virtually no limit to other components which may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the dendritic cells.

Adjuvants are substances that non-specifically enhance or potentiate the immune response (*e.g.*, CTLs) to an antigen, and would thus be considered useful in formulations of the present invention. For example, cholera toxin acts locally as a mucosal adjuvant for the induction of peptide-specific CTLs following intranasal immunization of dendritic cells with CTL epitope peptides (Porgador *et al.*, 1997;

Porgador *et al.*, 1998). Several immunological adjuvants (*e.g.*, MF59) specific for dendritic cells and their preparation have been described previously (Dupis *et al.*, 1998; Allison, 1997; Allison, 1998). The use of such adjuvants in the present invention are considered. In another embodiment of the present invention, cytokines are used in combination with the delivery of the p53 expression construct. Cytokines are secreted, low-molecular weight proteins that regulate the intensity and duration of the immune response by exerting a variety of effects on lymphocytes and other immune cells. Several cytokines have been directly linked to influencing dendritic cell migration to lymphoid tissues (*e.g.*, TNF-α), accelerating the maturation of dendritic cells into efficient antigen-presenting cells for T-lymphocytes (*e.g.*, GM-CSF, IL-1 and IL-4) (Dupis *et al.*, 1998; Allison, 1997; Allison, 1998; U.S. Patent 5,849,589, specifically incorporated herein by reference in its entirety) and acting as immunoadjuvants (*e.g.*, IL-12) (Gabrilovich *et al.*, 1996). The use of these and other cytokines (*e.g.*, FLT-3 ligand, CD 40) are considered in the present invention.

The formulation of pharmaceutically-acceptable excipients and carrier solutions are well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including, *e.g.*, intradermal, parenteral, intravenous, intramuscular, intranasal, and oral administration and formulation.

1. INJECTABLE COMPOSITIONS AND DELIVERY

The preferred method of the self gene adenovirus expression construct delivery to dendritic cells in the present invention is via intradermal injection. However, the pharmaceutical compositions disclosed herein may alternatively be administered parenterally, intravenously, intramuscularly, or even intraperitoneally as described in U.S. Patent 5,543,158; U.S. Patent 5,641,515 and U.S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Injection of self gene constructs and transduced dendritic cells may be delivered by syringe or any other method used for injection of a solution, as long as the expression construct or transduced cells can pass through the particular gauge of needle required for injection.

A novel needeless injection system has recently been described (U.S. Patent 5,846,233) having a nozzle defining an ampule chamber for holding the solution and an energy device for pushing the solution out of the nozzle to the site of delivery. A syringe system has also been described for use in gene therapy that permits multiple injections of predetermined quantities of a solution precisely at any depth (U.S. Patent 5,846,225).

Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also

be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

2. ORAL COMPOSITIONS AND DELIVERY

The pharmaceutical compositions disclosed herein may be delivered *via* oral administration to an animal. and as such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

The active compounds may even be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (Mathiowitz *et al.*, 1997; Hwang *et al.*, 1998; U.S. Patent 5,641,515; U.S. Patent 5,580,579 and U.S. Patent 5,792,451, each specifically incorporated herein by reference in its entirety). The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical

formulations, and as such, a variety of dosages and treatment regimens may be desirable.

For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally-administered formulation. For example, a mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an oral solution such as those containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, including: gels, pastes, powders and slurries, or added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants, or alternatively fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

3. ADDITIONAL MODES OF DELIVERY

In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of self gene delivery. Sonophoresis (*i.e.*, ultrasound) has been used and described in U.S. Patent 5,656,016 (specifically incorporated herein by reference in its entirety) as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U.S. Patent 5,779,708), microchip devices (U.S. Patent 5,797,898), ophthalmic formulations (Bourlais *et al.*, 1998), transdermal matrices (U.S. Patent 5,770,219 and U.S. Patent 5,783,208), rectal delivery (U.S. Patent 5,811,128) and feedback controlled delivery (U.S. Patent 5,697,899), each specifically incorporated herein by reference in its entirety.

G. MONITORING IMMUNE RESPONSE

In one embodiment of the present invention, self gene adenovirus vectors are intradermally administered to dendritic cells. Subsequently, the dendritic cells

express and present self gene antigens to immune effector cells, thereby stimulating an anti- self gene response. In another embodiment, the immune effector cells are cytotoxic T lymphocytes (CTLs). Thus, an important aspect of the invention is the ability to monitor immune responses, specifically CTLs.

5

1. CTL ASSAY

Cytotoxic T lymphocyte activity can be assessed in freshly isolated peripheral blood mononuclear cells (PBMC), in phytohaemaglutinin-stimulated IL-2 expanded cell lines established from PBMC (Bernard *et al.*, 1998) or by T cells isolated from 10 previously immunized subjects and restimulated for 6 days with DC infected with Adenovirus self gene using standard 6 h ^{51}Cr release microtoxicity assays. Colonic T-cells have been tested for their ability to mediate both perforin and Fas ligand-dependent killing in redirected cytotoxicity assays (Simpson *et al.*, 1998). The colon cytotoxic T lymphocytes displayed both Fas- and perforin-dependent killing. 15 Recently, an *in vitro* dehydrogenase release assay has been developed that takes advantage of a new fluorescent amplification system (Page *et al.*, 1998). This approach is sensitive, rapid, reproducible and may be used advantageously for mixed lymphocyte reaction (MLR). It may easily be further automated for large scale cytotoxicity testing using cell membrane integrity, and is thus considered in the 20 present invention. In another fluorometric assay developed for detecting cell-mediated cytotoxicity, the fluorophore used is the non-toxic molecule alamar blue (Nociari *et al.*, 1998). The alamarBlue is fluorescently quenched (*i.e.* low quantum yield) until mitochondrial reduction occurs, which then results in a dramatic increase in the alamarBlue fluorescence intensity (*i.e.* increase in the quantum yield). This 25 assay is reported to be extremely sensitive, specific and requires a significantly lower number of effector cells than the standard ^{51}Cr release assay.

2. ANTI-CTL ANTIBODIES

It is also contemplated in the present invention, that antibodies directed against 30 specific CTL epitopes may be used to assay CTL immune responses. The culturing and activation of mononuclear leukocytes with a standard stimulus known to activate

such cells has been described in U.S. Patent 5,843,689 (specifically incorporated herein by reference in its entirety). After culturing, aliquots of the cells are incubated with fluorophore-conjugated monoclonal antibodies to antigenic determinants of a particular mononuclear subclass (*e.g.*, CTLs). The incubated aliquots are analyzed on a flow cytofluorometer . It is contemplated that the use of CTL specific monoclonal antibodies and fluorophore-conjugated monoclonal antibodies (*e.g.*, CD8+, FasL, CD4+) will be of particular use as assays in the present invention.

H. *EX VIVO* PREPARATION OF DENDRITIC CELLS

In one embodiment of the present invention, a method for a p53-directed immune response in a subject is induced by: 1) obtaining dendritic cells from the subject, 2) infecting dendritic cells with an adenoviral vector comprising a p53 gene under the control of a promoter operable in eukaryotic cells and 3) the p53 adenovirus-infected dendritic cells are administered to the subject. It is contemplated that infected dendritic cells will present p53 antigens to immune effector cells and therefore stimulate an anti-p53 response in the subject. Thus, an important aspect of the present invention is to obtain dendritic cells from the subject or induce precursor cells (*e.g.*, monocytes) to differentiate into dendritic cells for infection with p53 adenoviral vectors for use in treatment of hyperproliferative disease.

It has been observed experimentally that patients with advanced stages of certain types of cancer have reduced function of dendritic cells (*i.e.* defective antigen presentation), but that these patients could give rise to functional dendritic cells through the *in vitro* growth and stimulation of stem cells (Gabrilovich *et al.*, 1997). The stem cells were obtained from the cancer patients, stimulated to differentiate into dendritic cells by the addition of granulocyte/macrophage colony-stimulating factor and IL-4. and observed to elicit much higher levels of CTL responses than mature dendritic cells obtained from the cancer patients (Gabrilovich *et al.*, 1997). Thus, it is contemplated in the present invention that stem cell precursor stimulated dendritic cell differentiation is used as a method for *ex vivo* treatment of hyperproliferative disease.

A method of culturing and inducing the differentiation of monocytes into dendritic cells has been described in U.S. Patent 5,849,589 (specifically incorporated herein by reference in its entirety). The method of monocyte differentiation into dendritic cells consists of a culture medium stimulated with GM-CSF, IL-4 and TNFα. An alternate method of isolating dendritic cells has been described by Cohen *et al.* (U.S. Patent 5,643,786, specifically incorporated herein by reference in its entirety). This method involves elutriating peripheral blood samples in at least four flow rates from an elutriation rotor. Calcium ionophore is used to stimulate monocytes isolated during the process into dendritic cells and treatment for diseases involving re-introduction of the activated dendritic cells are also disclosed. It is also possible to prepare immortalized precursor cells that is considered useful in the present invention (U.S. Patent 5,830,682; U.S. Patent 5,811,297, each specifically incorporated herein by reference in its entirety). In another example, an immature dendritic cell line derived from p53 growth suppressor gene deficient animals are prepared (U.S. Patent 5,648,219, specifically incorporated herein by reference in its entirety). The immature dendritic cell line may be induced to become an activated, immortalized dendritic cell line that will stimulate T-cell proliferation and is thus contemplated for use in the present invention. Methods and compositions for use of human dendritic cells to activate T-cells for immunotherapeutic responses against primary and metastatic prostate cancer have also been described (U.S. Patent 5,788,963, specifically incorporated herein by reference in its entirety). After the exposure of the dendritic cells to prostate cancer antigen *in vitro*, the dendritic cells are administered to a prostate cancer patient to activate T-cell responses *in vivo*. An important embodiment of the invention described above (U.S. Patent 5,788,963) is a method to extend the life span of the human dendritic cells by cryopreservation. This method may be of important utility in the present invention for long term storage of p53 adenoviral infected dendritic cells.

I. PHARMACEUTICALS AND METHODS OF TREATING CANCER

In a particular aspect, the present invention provides methods for the treatment of various hyperproliferative diseases. Treatment methods will involve treating an individual with an effective amount of a viral particle, as described above, containing a self gene of interest. An effective amount is described, generally, as that amount sufficient to detectably and repeatedly to ameliorate, reduce, minimize or limit the extent of the disease or its symptoms. More rigorous definitions may apply, including elimination, eradication or cure of disease.

To kill cells, inhibit cell growth, inhibit metastasis, decrease tumor or tissue size and otherwise reverse or reduce the malignant phenotype of tumor cells, using the methods and compositions of the present invention, one would generally contact a dendritic cell with the therapeutic expression construct. This may be combined with compositions comprising other agents effective in the treatment of hyperproliferative cells. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the expression construct and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the second agent.

Alternatively, the dendritic cell therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and expression construct are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several

d (2, 3, 4, 5, 6 or 7) to several wk (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

Various combinations may be employed, gene therapy is "A" and the radio- or chemotherapeutic agent is "B":

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B

B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

Administration of the therapeutic expression constructs of the present invention to a patient will follow general protocols for the administration of chemotherapeutics, taking into account the toxicity, if any, of the vector. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described dendritic cell therapy.

Aqueous compositions of the present invention comprise an effective amount of the compound, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions can also be referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is

contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. Also of import is the subject to be treated, in particular, the state of the subject and the protection desired. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently may be described in terms of plaque forming units (pfu) of the viral construct. Unit doses range from 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13} pfu and higher.

Preferably, patients will have adequate bone marrow function (defined as a peripheral absolute granulocyte count of > 2,000 / mm^3 and a platelet count of 100,000 / mm^3), adequate liver function (bilirubin < 1.5 mg / dl) and adequate renal function (creatinine < 1.5 mg / dl).

1. GENE THERAPY

One of the preferred embodiments of the present invention involves the use of viral vectors to deliver therapeutic genes to dendritic cells for the treatment of cancer. Cancer cells include cancers of the lung, brain, prostate, kidney, liver, ovary, breast, skin, stomach, esophagus, head and neck, testicles, colon, cervix, lymphatic system and blood. Of particular interest are non-small cell lung carcinomas including squamous cell carcinomas, adenocarcinomas and large cell undifferentiated carcinomas, tumor suppressors, antisense oncogenes, and inhibitors of apoptosis.

According to the present invention, one may treat the cancer by directly injection a tumor with the viral vector. Alternatively, the tumor may be infused or

perfused with the vector using any suitable delivery vehicle. Local or regional administration, with respect to the tumor, also is contemplated. Finally, systemic administration may be performed. Continuous administration also may be applied where appropriate, for example, where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease. Delivery *via* syringe or catherization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition *via* continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

For tumors of > 4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of < 4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals.

In certain embodiments, the tumor being treated may not, at least initially, be resectable. Treatments with therapeutic viral constructs may increase the resectability of the tumor due to shrinkage at the margins or by elimination of certain particularly invasive portions. Following treatments, resection may be possible. Additional viral treatments subsequent to resection will serve to eliminate microscopic residual disease at the tumor site.

A typical course of treatment, for a primary tumor or a post-excision tumor bed, will involve multiple doses. Typical primary tumor treatment involves a 6 dose application over a two-week period. The two-week regimen may be repeated one, two, three, four, five, six or more times. During a course of treatment, the need to complete the planned dosings may be re-evaluated.

2. CHEMOTHERAPY

Cancer therapies also include a variety of combination therapies with both chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin and methotrexate or any analog or derivative variant thereof.

3. RADIOTHERAPY

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell in a combined amount effective to kill the cell or prevent it from dividing.

J. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

MATERIALS AND METHODS

Animals

6-8 wk-old female BALB/c and CBA mice were purchased from Harlan Inc. (Indianapolis, IN) and were housed in specific pathogen-free units of the Division of Animal Care at Vanderbilt University Medical Center.

Reagents and cell lines

Tumor cells D459 were constructed by transfection of BALB/c 3T3 cells with EJ *ras* and a mutant human p53 expression vector. Details of this cell lines wore described elsewhere (Gabrilovich *et al.*, 1996; Yanuck *et al.*, 1993). MethA sarcoma cells were obtained from Dr. L. J. Old. This is a Ishida *et al.* transplantable 3-methylcholanthrene-induced sarcoma of BALB/c origin passaged as an ascitic tumor. P815 mouse mastocytoma cell lines transfected with mutant human p53 genes were also described elsewhere (Ciernik *et al.*, 1995). These two cell lines contain p53 genes with two different mutations, one in codon 135 (P815-135) and the other one in codon 173 (P8l5-173).

Control adenovirus (Ad-c) was prepared by deletion of E1 region from adenovirus serotype 5. Adenovirus containing human wild-type 53 (Ad-p53) was

obtained from Idtrogen Therapeutics Inc., Houston, TX. Recombinant mouse GM-CSF and IL-4 were obtained from R&D Systems, Minneapolis, MN.

FITC and PE labeled antibodies used in flow cytometry were purchased from Pharmigen (San Diego, CA): anti-CD11c (N418), anti-CD-86 (B7-2), anti-CD40 and anti-I-A^d. FITC- and PE-conjugated isotype matched IgG were used in controls. Anti-p53 antibodies were obtained from Dako Corporation, Capinteria, CA. FITC labeled anti-mouse Ig was obtained from Sigma, St. Louis, MO.

Cell preparation and infection with adenovirus

Bone marrow cells were prepared as described earlier (Gabrilovich *et al.*, 1996). Briefly, bone marrow cells were obtained from the femurs and tibias of BALB/c mice. Mononuclear cells were placed in tussie flasks at a concentration 5×10^5/ml in complete culture medium (CCM) (RPMI-1640, Gibco BRL, Gaithersburg, MD with 100 IU/ml penicillin, 0.1 mg/mL streptomycin, 1×10^5 M 2-mercaptoethanol and 10% fetal calf serum, HyClone, Logan, UT) supplemented with rmGM-CSF at a final concentration 3 ng/ml and rmIL-4 at a final concentration of 5 ng/ml. After 3 days, half of the medium was removed after gentle swirling and replenished with an equivalent amount of fresh GM-CSF and IL-4 supplemented medium. 3-4 days later, clusters of DC were dislodged. The purity of DC prepared in this fashion was greater than 60% at > 95% viability.

Splenic DC were prepared as described (Gabrilovich *et al.*, 1996). A single cell suspension was prepared by pressing the spleens through a wire mesh. Cells were then washed and incubated overnight in CCM. Non-adherent cells were layered onto a metrizamide (Nygaard, Oslo, Norway) gradient (14.5 g plus 100 ml RPMI 1640 medium) and centrifuged for 10 min at 600 g. Cells at the interface were washed once and resuspended in complete culture medium (CCM). DC's were identified by their distinctive morphology and by labeling with N418 (CD11c) antibody and had a purity >40% with >9S viability. T cells were isolated from lymph nodes using nylon wool columns as described elsewhere (Gabrilovich *et al.*, 1996).

10^6 DC obtained either from bone marrow or from spleen were infected with adenovirus at various multiplicities of infection (MOI) for 60 min in 1 ml of serum-free medium in 24-well plates. After that time, 1 ml of fresh medium supplemented with GM-CSF, IL-4 and 20% FCS was added. No IL-4 was added to splenic DC. Cells were incubated for another 24, 48, 72 or 120 h. After that time, cells were washed in PBS before use.

Tumor induction and immunization procedures

For immunization, bone marrow derived DC were used. Two hundred thousand dendritic cells were injected either iv, ip or sc into BALB/c mice. Two hundred thousand D459 cells or 6×10^5 MethA sarcoma cells were injected sc into the shaved backs of mice. These doses of tumor cells were chosen after preliminary studies showed that they resulted in tumor formation in 100% of the mice.

T cell proliferation assay

DCs infected with Ad-p53 or Ad-c were irradiated (2000 cGy) and added in triplicate to 5×10^4 T cells obtained from BALB/c mice immunized with Ad-p53 DC or, for a studies of allogeneic mixed leukocyte reaction (MLR), DCs were cultured with T cells obtained from CBA mice. After a 3 day incubation in 96 well U-bottomed plates, the cultures were pulsed with 1 μCi [^{3}H]thymidine (Amersham, Arlington Heights, IL) for 8-12 h. [^{3}H]Thymidine uptake was counted using a liquid scintillation counter.

Analysis of the p53 protein expression and expression of surface molecules

The efficiency of DC transduction was tested based on the overexpression of human p53 protein by FACS analysis. Briefly, DC after infection with Ad-p53 or Ad-c were fixed for 30 min with 2% parafarmaldehyde, permeabilized for 60 min with 0.2% Tween 20 and stained with anti-p53 antibody. FITC conjugated anti-mouse Ig was used as a secondary antibody. Non-specific binding was measured using secondary antibody alone. Cells were analyzed using flow cytometer

FACScalibur (Becton Dikinson, Mountain View, CA) with gates set around cluster of large cells. Expression of the surface molecules was studied on non-fixed, non-permeabilized DCs using monoclonal antibodies specific for B7-2, CD40, and IAd and analyzed by flow cytometry. Nonspecific binding was measured using isotype matched mouse Ig.

CTL assay.

T cell cytotoxicity was measured in a standard 6 h ^{51}Cr release assay. Briefly, 2×10^6T cells isolated from immunized mice were restimulated for 6 days with 2×10^5 splenic DC infected either with Ad-p53 or Ad-c in 24-well plates. Effector lymphocytes were incubated in duplicate with ^{51}Cr labeled target cells. Supernatants were harvested with a Skatron Harvesting System (Skatron, Norway) and radioactivity was counted on a gamma counter. The percent specific lysis was calculated as 100 × [(experimental release - spontaneous release) / maximum release - spontaneous release)].

EXAMPLE 2

DETERMINATION OF EFFECTIVE AD-P53 DOSES FOR DENDRITIC CELLS TRANSDUCTION

In preliminary studies, the most effective dose of Ad-p53 was determined. Ad-p53 and Ad-c at doses of 50-200 MOI did not significantly affect DC viability, which remained >95%. Higher doses of virus resulted in significant loss of viability (less than 50% at doses more than 500 MOI). The efficiency of transduction was estimated using intracellular staining with an anti-p53 antibody. The maximum level of p53 was detected at an Ad-p53 MOI of 100 pfu/cell. At this dose 40-45% DC were positive for p53 (FIG. 1). This dose of adenovirus was used in all subsequent studies. A 48 h incubation with Ad-p53 resulted in the highest levels of p53 protein, which slightly decreased 24 h later, and was undetectable by day 5. Thus, these preliminary studies demonstrated that Ad-p53 at a dose of 100 MOI was non-toxic for DC, and that Ad-p53-transduced DC expressed detectable levels of p53 protein. Infection of

DC with adenovirus did not affect the ability of these cells to stimulate allogeneic T cells, and slightly increased expression of B7-2 and CD40 molecules on their surface.

EXAMPLE 3

DETERMINING THE EFFICACY OF IMMUNIZATION WITH AD-P53 TRANSDUCED DC IN ELICITING AN ANTI-P53 IMMUNE RESPONSE AND HOW MANY IMMUNIZATIONS ARE REQUIRED TO ACHIEVE THE EFFECT

Mice were immunized with 2×10^5 DC infected 48 h before with either Ad-p53 or Ad-c. Three routes of immunization were tested (sc, ip and iv) and immune responses were assayed using 5 different target tumors: P815 cells, P815 cells infected with control adenovirus (P815-Ad-c), P815 cells infected with Ad-p53 (P815-Ad-p53), P815-135 cells and P815-173 cells. Mice were immunized once or twice with a two wk interval. Ten to 14 days after the last immunization, T cells were isolated and restimulated with Ad-p53 infected splenic DC. No CTLs were detected after a single immunization using any of the tested routes of immunization. However, two immunization resulted in significant CTL responses (FIG. 2A). The highest response was observed against Ad-p53 infected P815 cells, however significant responses was also seen using P815-135 and P815-173 as targets. It is important to note the very low CTL responses detected against P815 cells infected with the control adenovirus. In three studies performed no differences in the level of anti-p53 specific CTL responses were found between the different routes of immunization

EXAMPLE 4

IMMUNIZATION WITH AD-P53 DC RESULTS IN CTL RESPONSES AGAINST MUTANT MURINE P53

The studies described so far were performed using constructs and tumor cell lines expressing human p53. Since there is a high homology between human and murine p53, the inventors contemplated whether immunization with Ad-p53 DC also would result in CTL responses against overexpressed mutant murine p53. For these

studies, the murine MethA sarcoma tumor was used, a carcinogen-induced tumor bearing different point mutations in each allele of its endogenous p53 genes. Tumor cells were preincubated for 3 days with 50U/ml recombinant murine IFNγ and then used as a target in CTL assay. Low but clearly significant CTL responses specific for MethA were detected in mice immunized with Ad-p53 DC (FIG. 2B). Also tested was whether Ad-p53 DC were able to stimulate T cell proliferation in this system. T cells were obtained from immune mice (two immunizations with Ad-p53 DC) and were cultured with either uninfected DC (background level), or DC infected with Ad-c with Ad-p53. DC infected with Ad-p53, but not those infected with Ad-c were able to stimulate T cell proliferation significantly higher than background levels (FIG. 2C).

EXAMPLE 5

CTL AND T-CELL INDUCED IMMUNE RESPONSES PROVIDE TUMOR PROTECTION

Mice were immunized twice iv with Ad-p53 and Ad-c infected DC. 10 days after the second immunization they were challenged with either D459 tumor, bearing a mutant human p53 gene, or with MethA sarcoma cells, expressing mutant murine p53. Doses of tumor cells were selected which resulted in tumor formation in 100% of non-immune control mice. After immunization with Ad-p53 DC, 17 out 20 (85%) immunized mice were completely protected against D459 tumor and 8 out 11 mice (72.7%) were protected against MethA sarcoma (FIG. 3).

The inventors then investigated the effect of treatment of established poorly immunogeneic tumors with repeated injections with Ad-p53 infected DC. 2×10^5 D459 were inoculated sc. When tumors became palpable, treatment with Ad-p53 DC was initiated. Mice were immunized three times and tumor growth was observed for 7 wk. Treatment with Adp53 infected DC significantly slowed down the tumor growth (FIG. 4). Mice in this group were sacrificed due to do bulky tumor more than two wk later than mice in the control group.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS:

1. A method for treating a subject with a hyperproliferative disease comprising the steps of:

(i) identifying a subject with a hyperproliferative disease characterized by alteration or increased expression of a self gene product in at least some of the hyperproliferative cells in said patient; and

(ii) intradermally administering to said subject an expression construct comprising a self gene under the control of a promoter operable in eukaryotic dendritic cells,

whereby said self gene product is expressed by dendritic cells and presented to immune effector cells, thereby stimulating an anti-self gene product response.

2. The method of claim 1, wherein said self-gene product is an oncogene.

3. The method of claim 2, wherein said oncogene is selected from the group consisting of tumor suppressors, tumor associated genes, growth factors, growth-factor receptors, signal transducers, hormones, cell cycle regulators, nuclear factors, transcription factors and apoptic factors.

4. The method of claim 3, wherein said tumor suppressor is selected from the group consisting of Rb, p53, p16, p19, p21, p73, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC , PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2 and WT-1.

5. The method of claim 3, wherein said growth-factor receptor is selected from the group consisting of FMS, ERBB/HER, ERBB-2/NEU/HER-2, ERBA, TGF-β receptor, PDGF receptor, MET, KIT and TRK.

6. The method of claim 3, wherein said signal transducer is selected from the group consisting of SRC, ABl, RAS, AKT/PKB, RSK-1, RSK-2, RSK-3, RSK-B, PRAD, LCK and ATM.

7. The method of claim 3, wherein said transcription factor or nuclear factor is selected from the group consisting of JUN, FOS, MYC, BRCA1, BRCA2, ERBA, ETS, EVII, MYB, HMGI-C, HMGI/LIM, SKI, VHL, WT1, CEBP-α, NFKB, IKB, GL1 and REL.

8. The method of claim 3, wherein said growth factor is selected from the group consisting of SIS, HST, INT-1/WT1 and INT-2.

9. The method of claim 3, wherein said apoptic factor is selected from the group consisting of Bax, Bak, Bim, Bik, Bid, Bad, Bcl-2, Harakiri and ICE proteases.

10. The method of claim 3, wherein said tumor associated gene is selected from the group consisting of CEA, mucin, MAGE and GAGE.

11. The method of claim 4, wherein said tumor suppressor product is p53.

12. The method of claim 1, wherein said expression construct is a viral vector.

13. The method of claim 12, wherein said viral vector is an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector, an alphavirus vector, or a herpesviral vector.

14. The method of claim 13. wherein said viral vector is an adenoviral vector.

15. The method of claim 14, wherein said adenoviral vector is replication-defective.

16. The method of claim 15, wherein the replication defect is a deletion in the E1 region of the virus.

17. The method of claim 16, wherein the deletion maps to the E1B region of the virus.

18. The method of claim 17, wherein the deletion encompasses the entire E1B region of the virus.

19. The method of claim 18, wherein the deletion encompasses the entire E1 region of the virus.

20. The method of claim 1, wherein said promoter is selected from the group consisting of CMV IE, human or murine dectin-1, human or murine dectin-2, human CD11c, mammalian F4/80 and human or murine MHC class II.

21. The method of claim 20, wherein said promoter is CMV IE.

22. The method of claim 1, wherein said expression vector further comprises a polyadenylation signal.

23. The method of claim 1, wherein said hyperproliferative disease is cancer.

24. The method of claim 23, wherein said cancer is selected from the group consisting of lung, head, neck, breast, pancreatic, prostate, renal, bone, testicular, cervical, gastrointestinal, lymphoma, brain, colon, skin and bladder.

25. The method of claim 1, wherein said hyperproliferative disease is selected from the group consisting of RA, IBD, OA, leiomyomas, adenomas, lipomas, hemangiomas, fibromas, melanomas, restenosis, pre-neoplastic lesions in the lung and psoriasis.

26. The method of claim 1, wherein said expression construct is administered via injection.

27. The method of claim 26, further comprising multiple injections.

28. The method of claim 26, wherein the injection is performed local to a hyperproliferative or tumor site.

29. The method of claim 26, wherein the injection is performed regional to a hyperproliferative or tumor site.

30. The method of claim 26, wherein the injection is performed distal to a hyperproliferative or tumor site.

31. The method of claim 1, wherein intradermal administration is via continuous infusion.

32. The method of claim 1, wherein said subject is a human.

33. The method of claim 1, wherein said immune effector cells are CTLs.

34. The method of claim 1, further comprising administering to said subject at least a first cytokine.

35. The method of claim 34, further comprising administering to said subject a second cytokine, different from said first cytokine.

36. The method of claim 34, wherein said cytokine is selected from the group consisting of GM-CSF, IL-4, C-KIT, Steel factor, TGF-β, TNF-α and FLT3 ligand.

37. The method of claim 34, wherein said cytokine is administered as a gene encoded by said expression construct.

38. A method for treating a pathogen-induced disease in a subject comprising the steps of:

(i) identifying a subject with a pathogen-induced disease characterized by alteration or increased expression of a pathogen gene product in at least some of the pathogen-induced cells in said patient;

(ii) intradermally administering to said subject an expression construct comprising a pathogen gene under the control of a promoter operable in eukaryotic dendritic cells;

(ii) infecting said dendritic cells with an adenoviral vector comprising a pathogen gene product under the control a promoter operable in eukaryotic cells; and

(iii) administering the adenovirus-infected dendritic cells to said subject,

whereby said pathogen gene product is expressed by dendritic cells and presented to immune effector cells, thereby stimulating an anti-pathogen gene product response.

39. The method of claim 38, wherein said dendritic cells are obtained from peripheral blood progenitor cells.

40. The method of claim 38, further comprising multiple administrations of adenovirus-infected dendritic cells.

41. The method of claim 38, wherein said pathogen is selected from the group consisting of bacterium, virus, fungus, parasitic worm, amoebae and mycoplasma.

42. The method of claim 41, wherein said bacterium is selected from the group consisting of richettsia, listeria and histolytica.

43. The method of claim 41, wherein said virus is selected from the group consisting of HIV, HBV, HCV, HSV, HPV, EBV and CMV.

44. The method of claim 41, wherein said fungus is selected from the group consisting of hitoplasma, coccidis, immitis, aspargillus, actinomyces, blastomyces, candidia and streptomyces.

45. The method of claim 38, wherein said expression construct is a viral vector.

46. The method of claim 45, wherein said viral vector is an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector, an alphavirus vector, or a herpesviral vector.

47. The method of claim 46, wherein said viral vector is an adenoviral vector.

48. The method of claim 47, wherein said adenoviral vector is replication-defective.

49. The method of claim 48, wherein the replication defect is a deletion in the E1 region of the virus.

50. The method of claim 49, wherein the deletion maps to the E1B region of the virus.

51. The method of claim 50, wherein the deletion encompasses the entire E1B region of the virus.

52. The method of claim 51, wherein the deletion encompasses the entire E1 region of the virus.

53. The method of claim 38, wherein said promoter is selected from the group consisting of CMV IE, human or murine dectin-1, human or murine dectin-2, human CD11c, mammalian F4/80 and human or murine MHC class II.

54. The method of claim 53, wherein said promoter is CMV IE.

55. The method of claim 38, wherein said expression vector further comprises a polyadenylation signal.

56. The method of claim 38, wherein intradermal administration is by injection of the expression construct.

57. The method of claim 56, further comprising multiple injections.

58. The method of claim 56, wherein the injection is performed local to a pathogen-induced disease site.

59. The method of claim 56, wherein the injection is performed regional to a pathogen-induced disease site.

60. The method of claim 56, wherein the injection is performed distal to a pathogen-induced disease site.

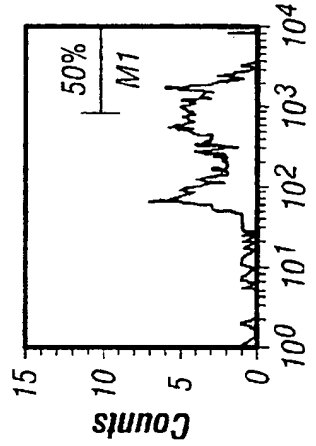

FIG. 1C

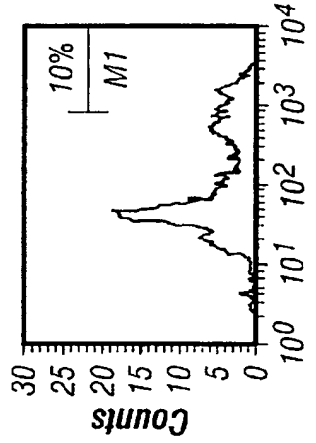

FIG. 1B

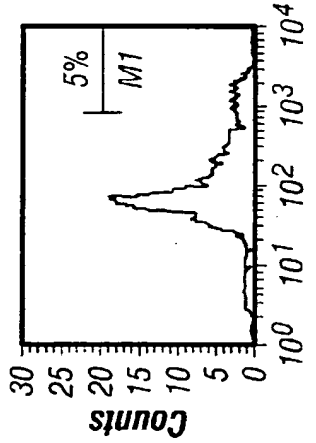

FIG. 1A

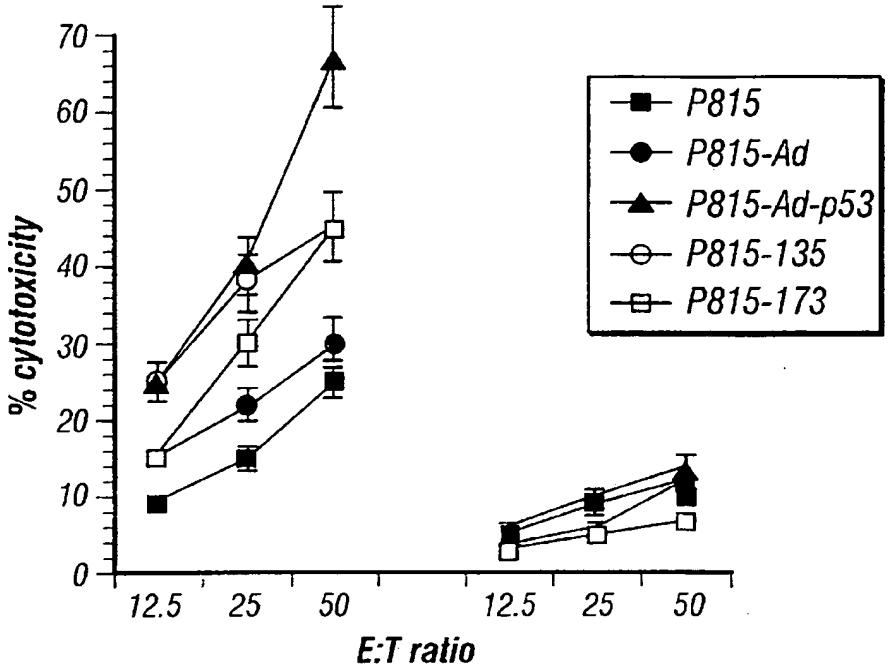
70
60
50
40
30
20
10
0
% cytotoxicity
12.5
25
50
12.5
25
50
E:T ratio
P815
P815-Ad
P815-Ad-p53
P815-135
P815-173

FIG. 2A

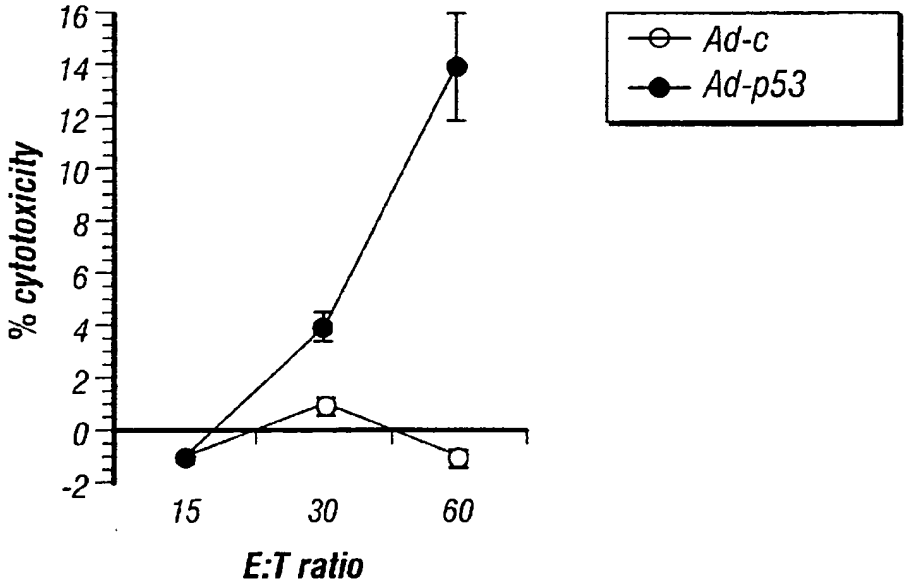
16
14
12
10
8
6
4
2
0
-2
% cytotoxicity
15
30
60
E:T ratio
Ad-c
Ad-p53

FIG. 2B

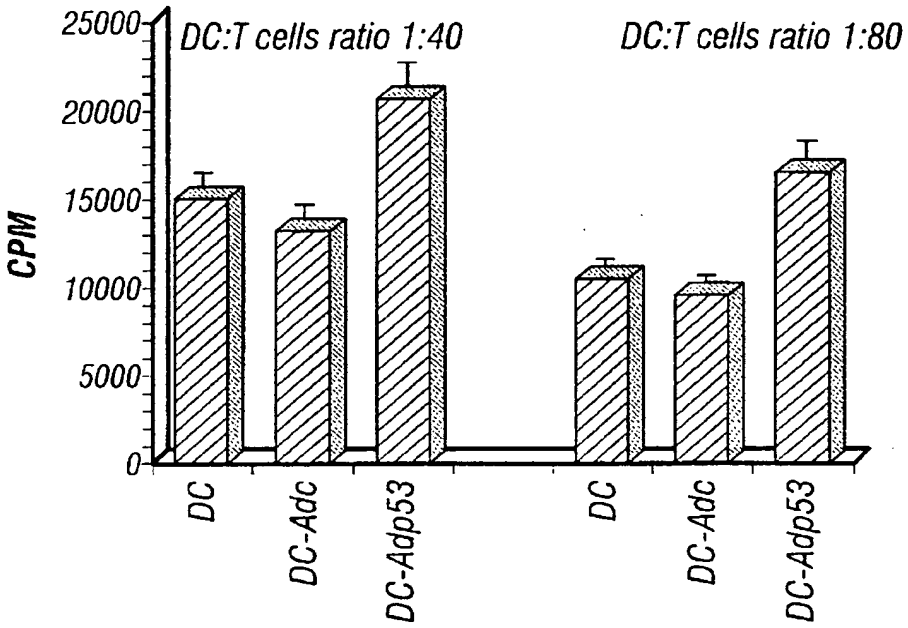
DC:T cells ratio 1:40
DC:T cells ratio 1:80
CPM
25000
20000
15000
10000
5000
0
DC
DC-Adc
DC-Adp53
DC
DC-Adc
DC-Adp53

FIG. 2C

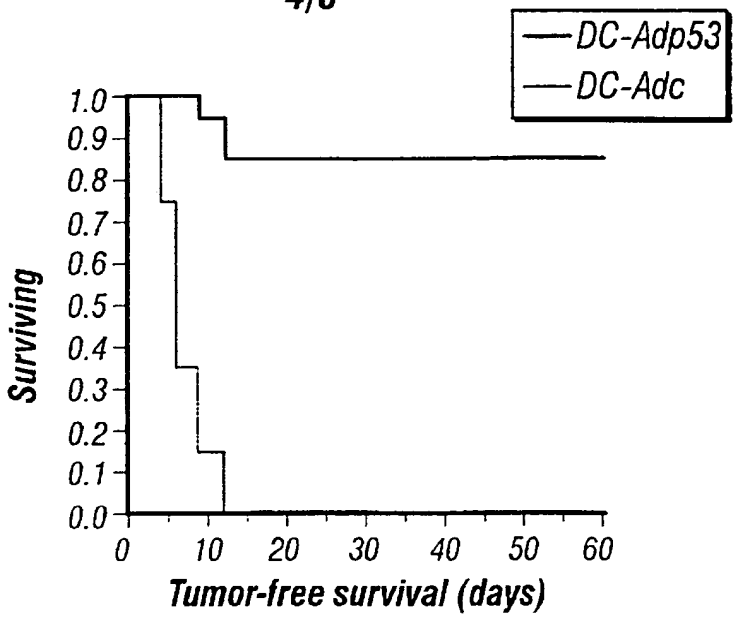
DC-Adp53
DC-Adc
Surviving
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
Tumor-free survival (days)

FIG. 3A

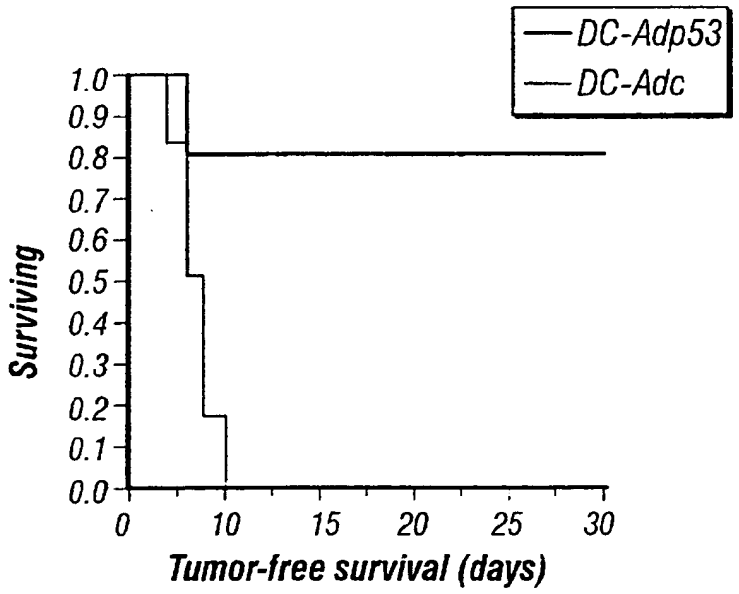
DC-Adp53
DC-Adc
Surviving
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
15
20
25
30
Tumor-free survival (days)

FIG. 3B

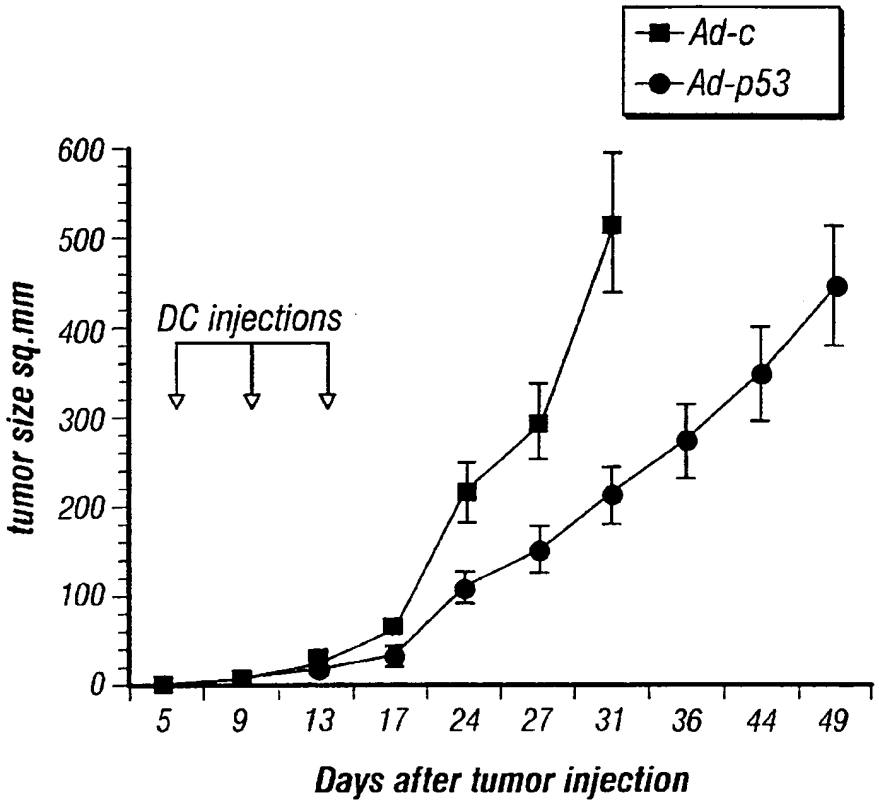
Ad-c
Ad-p53
600
500
400
300
200
100
0
tumor size sq.mm
DC injections
5
9
13
17
24
27
31
36
44
49
Days after tumor injection

FIG. 4

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 September 2000 (21.09.2000)

PCT

(10) International Publication Number
WO 00/54839 A3

(51) International Patent Classification[7]: A61K 48/00

(21) International Application Number: PCT/US00/07055

(22) International Filing Date: 15 March 2000 (15.03.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/124,482 15 March 1999 (15.03.1999) US
60/124,388 15 March 1999 (15.03.1999) US

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(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
25 January 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/54839 A3

(54) Title: DENDRITIC CELLS TRANSDUCED WITH A WILD-TYPE SELF GENE ELICIT POTENT ANTITUMOR IMMUNE RESPONSES

(57) Abstract: The present invention relates to immunotherapy methods for treating hyperproliferative disease or pathogen-induced diseases in humans. More specifically, the invention is directed, in one embodiment, to methods for treating a subject with a hyperproliferative disease in which the expression of a self gene is upregulated in hyperproliferative cells. In another embodiment, an adenoviral expression construct comprising a self gene under the control of a promoter operable in eukaryotic cells is intradermally administered to said hyperproliferative cells. In another embodiment of the present invention, a pathogen-induced disease in which the pathogen gene expression is increased or altered, is treated by intradermally administered a pathogen gene under the control of a promoter operable in eukaryotic cells. The present invention thus provides immunotherapies for treating hyperproliferative and pathogen diseases by attenuating the natural immune systems CTL response against hyperproliferative cells or overexpressing mutant p53 antigens.

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 00/07055

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 99 47180 A (SHANKARA SRINIVAS ;ARMENTANO DONNA (US); GENZYME CORP (US); ROBERT) 23 September 1999 (1999-09-23) * see abstract, pages 7,11,21 and 25-26 * ---	1-8, 10-23, 26, 28-30, 32,33
X	WO 98 06863 A (NELSON PETER J ;US HEALTH (US); NELSON EDWARD L (US)) 19 February 1998 (1998-02-19) * see abstract, table 1, pages 19-20 and 22-24 * --- -/--	1-8,10, 11, 23-26, 28-30, 32-34, 36,37

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 July 2000	17.10.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gore, V

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HURPIN C ET AL: "The mode of presentation and route of administration are critical for the induction of immune responses to p53 and antitumor immunity" VACCINE,GB,BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 16, no. 2/03, January 1998 (1998-01), pages 208-215, XP002098284 ISSN: 0264-410X * see abstract, page 209 and page 210 right col. * ---	1-8, 10-24, 32,33
X,P	WO 99 26662 A (PASTEUR MERIEUX SERUMS VACC ;HAENSLER JEAN (FR); ERDILE LORNE (US)) 3 June 1999 (1999-06-03) * see claims 1,2,5 and 8, pages 3-4 * ---	1-8,10, 11, 20-24, 32,33
X,P	WO 99 27958 A (ENSOLI BARBARA ;IST SUPERIORE SANITA (IT)) 10 June 1999 (1999-06-10) * see claims 1,5,18-22,37 and pages 10-11* ---	1,26, 28-30, 32-34, 36,37
X	WO 97 00954 A (UNIV TEXAS ;HSIEH JER TSONG (US); LIN SUE HWA (US)) 9 January 1997 (1997-01-09) * see pages 4,30,43,54 and table 3 * ---	1-8, 10-24, 32,33
X	YONGHONG WAN ET AL: "DENDRITIC CELLS TRANSDUCED WITH AN ADENOVIRAL VECTOR ENCODING A MODEL TUMOR-ASSOCIATED ANTIGEN FOR TUMOR VACCINATION" HUMAN GENE THERAPY,XX,XX, vol. 8, no. 11, 20 July 1997 (1997-07-20), pages 1355-1363, XP000877349 ISSN: 1043-0342 * see abstract, page 1358 right col., page 1356 right col. and page 1362 last paragraph * ---	1-3, 12-24, 26,28, 32,33
Y	WO 97 29183 A (US HEALTH ;HWU PATRICK (US); REEVES MARK (US); ROSENBERG STEVEN A) 14 August 1997 (1997-08-14) * see abstract, cl. 16,46-47, pages 4,6,20-21,27, 30 and 32 * --- -/--	1-37

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 00/07055

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DELEO ET AL.: "P53-BASED IMMUNOTHERAPY OF CANCER" CRITICAL REVIEWS IN IMMUNOLOGY,XX,CRC PRESS, INC, vol. 18, no. 1/02, 1998, pages 29-35, XP000877346 ISSN: 1040-8401 * see abstract, page 32 right col. and page 33 *	1-37
Y	WO 97 03703 A (RHONE POULENC RORER PHARMA ;PHILIP RAMILA (US); LEBKOWSKI JANE S () 6 February 1997 (1997-02-06) * see pages 6-9,11,13,54 and cl.26 *	1-37
A	RAZ E ET AL: "INTRADERMAL GENE IMMUNIZATION: THE POSSIBLE ROLE OF DNA UPTAKE IN THE INDUCTION OF CELLULAR IMMUNITY TO VIRUSES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 91, no. 20, 27 September 1994 (1994-09-27), pages 9519-9523, XP002021792 ISSN: 0027-8424 * see abstract, pages 9519 and 9523 *	1-37

3

INTERNATIONAL SEARCH REPORT

ɜrnational application No.
PCT/US 00/07055

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 - 37

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-37

Method for treating hyperproliferative diseases comprising administering a vector expressing a self gene

2. Claims: 38-60

Method for treating a pathogen-induced disease comprising administering a vector expressing a pathogen gene

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/07055

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WO 9806863 A	19-02-1998	AU 3980497 A	06-03-1998
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